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Se eu vi mais longe, foi por estar de pé sobre ombros de gigantes.

(Si j'ai vu plus loin c'est en me tenant sur les épaules de géants.)

Isaac Newton

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INTRODUCTION GENERALE

Le rhumatisme articulaire aigu (RAA) est une maladie inflammatoire résultant des infections supérieures non suppuratives causées par streptocoques β-hémolytique du groupe A (Sβ-HA). Dans sa forme classique, il se produit comme une maladie aiguë, febrile et auto-limitée. Ses effets touchent principalement les tissus conjonctifs, notamment le cœur et les articulations et peut entraîner des blessures chronique et progressive aux valves cardiaques entraînant une insuffisance cardiaque et même la mort.

La pharyngite causée par la Sβ-HA est l'infection la plus fréquente chez les enfants, en particulier dans les pays en voie de développement. Au Brésil, par exemple, la prévalence de la RAA est de 3-5% chez les enfants et les adolescents. Six décennies plus tôt le traitement de choix pour ces infections recommandé par l'American Academy of Pedriatrics, American Heart Association, et par la Organisation Mondiale de la Santé était un schéma thérapeutique basé sur des injections mensuelles de 1,2 million d'unités internationales (UI) de la pénicilline G benzathine (PenGB) (1, 3, 4, 5). Cependant, certains échecs dans la prophylaxie de la RAA ont indiqué les défaillances dans ce système (1, 4,7,8). Le succès limité de cette thérapie serait dû aux plusieurs facteurs tels que les complications chez les malades, l'inflammation chronique dans les amygdales des tissus qui peuvent empêcher la pénétration du médicament et de la coexistence de bactéries produisant la penicillinase (une enzyme qui inactive la pénicilline). Quelques études pharmacocinétiques comparant les différentes préparations de médicaments contenant de PenGB ont conclu que des variations dans les propriétés physiques telles que le degré de dissolution, la viscosité de la préparation, la taille des cristaux affecte le taux de libération du médicament à partir du site d'injection (1, 12) provoquant ainsi des modifications dans la biodisponibilité du médicament.

La concentration minimale inhibitrice (CMI) de PenGB contre les Sβ-HA est estimée entre 10-30 ηg/mL (1). Certaines études ont montré, 21 jours après l'injection, des niveaux

dans le sérum et aux amygdales de moins de 20 ηg/mL, ce qui pourrait indiquer d'éventuelles insuffisances du régime en tant que prophylaxie pour RAA. Ces études suggèrent que des doses plus élevées du médicament ou la diminution de l'intervalle entre les doses peuvent être plus appropriées, en particulier dans les populations à risque élevé (4, 7). L'adhésion des patients au traitement prophylactique de quatre semaines est très faible ; les raisons sont multifactorielles et comprennent les difficultés d'accès aux services de santé, les différences culturelles dans la perception de la maladie, et même les manifestations cliniques causées par le RAA.

Des systèmes pharmaceutiques conçus pour permettre un effet thérapeutique prolongé par la libération continue du médicament pendant une longue période de temps peut être une alternative efficace aux problèmes de la prophylaxie du RAA. Certains avantages thérapeutiques tels que la fréquence réduite de l'administration, une biodisponibilité accrue, réduisant ainsi la quantité totale de médicament administré, un meilleur contrôle de l'absorption du médicament et une réduction des effets secondaires, peuvent être garantis par de tels systèmes. La nanothérapie propose des systèmes transporteurs de médicaments capables d'amener des médicaments spécifiquement dans les tissus affectés, garantissant une stabilité suffisante, une meilleure absorption, à libération contrôlée et par conséquent l'activité pharmacologique attendu. Parmi ces systèmes se trouvent les microémulsions (ME) et les nanoémulsions (ηE), qui sont essentiellement des émulsions avec la taille des gouttelettes de moins de 1μm à des avantages en termes de propriétés biologiques et les médicaments tels que la biodégradabilité, la biocompatibilité, la stabilité physique et la facilité de la production (9). Ces systèmes sont également utilisés dans les systèmes à libération contrôlée (SLC), formant un dépôt de médicaments après l'injection. L'utilisation des ME et ηE comme SLC pour la PenGB pourrait permettre une utilisation plus rationnelle des antibiotiques et d'améliorer l'adhésion des patients au traitement du rhumatisme articulaire aigu.

L'objectif de ce travail était d'étudier un nanosystème emulsioné pour la PenGB à fin de developer un système pour améliorer le traitement de le rhumatisme articulaire aigu. Comme les systèmes fournissent la libération contrôlée, des petites quantités du principe actif sont suffisantes pour atteindre le CIM. Pour analyser cette libération et pour faire des contrôles pendant le développement et production des systèmes, il est nécessaire de disposer des méthodes de dosage sensibles et fiables. Dans une première partie (bibliographique), nous étudierons les caractéristiques de la maladie. Nous aborderons dans cette partie les aspects qui concerne l'épidémiologie, les aspects cliniques, les traitments avec leurs possibles incovenients. Encore dans la partie bibliographique, nous passerons en revue les nouvelles conceptes sur les systèmes emulsionées dans l'époque de la nanotechonologie. En particulier, les différences thèoriques et technologiques entre des microèmulsions et nanoémulsions seront abordées.

Dans la deuxième partie (travaux expérimentaux analytiques), la mise au point et la validation des techniques d'analyse de PenGB seront décrites.

Dans la troisième partie (travaux expérimentaux pharmacotechnologiques), la mise en œuvre des nanosystèmes de PenGB sera décrite. Les propriétés physico-chimiques de ces systèmes seront évaluées et l'activité microbiologique de la PenGB incorporé dans ces vecteurs será déterminé *in vitro*.

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Premier Partie

TRAVAUX ANTERIEURS

TITLE PAGE

TRENDS IN RHEUMATIC FEVER: CLINICAL ASPECTS AND PERSPECTIVES IN THE PROPHYLATIC TREATMENTS

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Short title**: Trends in rheumatic fever**

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ABSTRACT

Acute rheumatic fever (ARF) or rheumatic fever (RF), a systemic illness that may occur following Group A beta-haemolytic streptococcal (GABHS) pharyngitis in children, is a major problem in countries with limited resources. It affects the cardiac valves and muscles, joints, skin and central nervous system. Preventive and prophylactic therapy is indicated to avoid further valve damage. Primary prophylaxis (an initial course of antibiotics administered to eradicate the streptococcal infection) also serves as the first course of secondary prophylaxis: an injection of Benzathine Penicillin G (BPG) suspension every 3 or 4 weeks. Despite its excellent *in vitro* efficacy, the inability of penicillin to eradicate GABHS is frequently reported. Over the past 50 years, the rate of penicillin failure has consistently increased from about 7% in 1950 to almost 40% in 2000. Nevertheless, penicillin is still used for treatment of ARF, despite its high failure rate, mainly because of its long track record and low cost. The aim of this work was to study the possible causes of failure, as well as the inconvenience of the current prophylactic treatment of ARF and suggest a new pharmacotherapeutic system that could replace the current one. The poor penetration into the tonsilar tissues is one of the major reasons for the failure of penicillin. Other explanations relate to (i) the bacterial interactions between GABHS and other members of the pharyngo-tonsillar bacterial flora; (ii) resistance or tolerance to the antibiotic used; (iii) inappropriate dose or drug delivery, (iv) duration of therapy, and (v) poor compliance, among others. Further pharmacokinetic studies reported that intramuscular injection of benzathine Penicillin G did not, in a significant proportion of patients, produce serum values above the minimal inhibitory concentration by week 3. Hence, this apparent failure of a month long schedule is not sufficient to successfully prevent ARF. Nanocarrier-based systems are able to confer stability, improved absorption, controlled and quantitative release on the encapsulated molecule and therefore, improve its pharmacodynamic activity. Site-specific delivery is designed to minimise undesired effects caused by conventional therapy Microemulsions have been shown to labile drug, control drug release, increase bioavailability and reduce the

variability of patient outcomes. The advantages for drug delivery offered by microemulsions include improved drug solubilization and protection against enzymatic hydrolysis, as well as the potential for enhanced absorption provided by surfactant-induced membrane fluidity leading to permeability changes. Microemulsions have great potential as a parenteral vehicle for Penicillin in the treatment of ARF because they may be used for intravenous, subcutaneous or intramuscular administration. Furthermore, they can be used to obtain prolonged release formulations. These systems can also modify the pharmacokinetics of the encapsulated drug, and that it significantly increase the half-life, the area under curve and the mean residence time. Therefore the delivery of penicillin G in a nanocarrier-based system, particularly a microemulsion, could be a plausible and innovative alternative to the current treatment.

Key –words: Penicillin G, Rheumatic Fever, Nanotechnology

INTRODUCTION

RF is a multisystem inflammatory disease process that follows upper airway infection with GABHS and is almost certainly of autoimmune origin. It is a non-suppurative sequel of Group A streptococcal pharyngitis, but the exact mechanisms by which it occurs remain to be elucidated. RF is said to “lick the joints and bite the heart”. This underlines the fact that cardiac involvement is the most serious manifestation of RF. An acute attack of RF may be associated with severe heart failure, which can be life-threatening if appropriate medical and surgical therapy is not instituted [1-3].

Considering the severity of the conditions associated with RF, it is important to diagnose it quickly and accurately. This diagnosis is based on clinical findings and supportive laboratory studies. The current trend in the diagnosed cases is an aspect that requires further attention. Although the incidence of RF has declined significantly since its peak in the 1940s [4], it still remains a major cause of acquired heart disease in developing countries. However, in the developed world, only 3% of streptococcal pharyngitis patients develop RF [5]. RF is now a rare disease in America. The high prevalence of RF in developing communities is undoubtedly related to poverty and overcrowding, factors which favor the transmission of streptococci, and its reduction in more well-to-do populations has been linked to improved living conditions and hygiene [4, 5].

Primary and secondary prophylaxis for the disease is relatively straightforward, consisting of patient education and the use of BPG. In the countries where the disease is most prevalent, antibiotic regimes for primary and secondary prevention can be effective and a vaccine may ultimately be developed, but it is unfortunate that the most important step in the eradication of RF - the improvement in living conditions and reduction of overcrowding - requires means that these countries cannot afford [6]. Therefore, RF accounts for most cases of acquired heart disease in children and young adults, causing

considerable suffering, serious disability and premature death. The social impact, in terms of hospitalization costs and clinic visits, is significant.

Most of the countries in which RF is prevalent do not have the financial resources or the sophisticated medical services required to treat the long-term sequelae of chronic valvular heart disease either. [6].

Accurate identification and treatment of tonsillopharyngitis caused by GABHS, with lifetime follow-up of patients with a history of RF, continues to be an important strategy for control of the disease [7, 8]. Since RF remains endemic in many countries where it is a major cause of morbidity and mortality, it is vital to understand the epidemiology, clinical aspects, and treatment strategies for this disease process [7, 9]. This will be the focus of the next sections.

EPIDEMIOLOGY

Epidemiological studies show that 15 million cases of streptococcal pharyngitis occur annually in the USA alone, resulting in an estimated US$2 billion of direct healthcare costs. The GABHS is among the most ubiquitous and versatile of human bacterial pathogens [10]. Although GABHS can cause serious invasive disease, pharyngitis is, by far, the most common infection. Despite decades of research, however, our knowledge of the precise molecular events mediating GABHS pharyngitis remains rudimentary [11, 12].

In a five-state laboratory and population-based surveillance study between 1995 and 1999, invasive GABHS infections were found to have occurred annually in the USA at a rate of 3·6 per 100 000 population, accounting for 9600–9700 cases and 1100–1300 deaths. Case-fatality ratios for pneumonia, necrotizing fasciitis, and central nervous system infections exceeded 20%, while the ratio for streptococcal toxic shock syndrome was 44·5%. The propensity of GABHS to elicit two delayed, non-suppurative sequels is remarkable: ARF and acute post-streptococcal glomerulonephritis. The latter is beyond the scope of this review, which focuses on RF [13].

The advent of antibiotics in the 1950s accelerated the decline of RF in developed countries in the second half of the 19th century [14, 15]. For example, in the USA the incidence of RF has fallen from 100 per 100,000 of population at the turn of the century to 45~55 per 100,000 between 1935 and 1960; 0,23 and 1,88 per 100,000 currently in the Japan and Denmark. In France the incidence is 0.08-0.15 per 100,000 among children of 5 – 14 years[16]. This contrasts sharply with the reported incidence in the developing world, which has been reported to be as high as 21 per 1000 [2]. However, according to the World Health Organization (WHO), about 0.5 million individuals acquire RF each year and most are in developing countries. However, epidemiological data from many developing countries is limited and the incidence is very likely to be under-estimated [2].

The incidence of RF is said to exceed 50 per 100,000 children. In many countries such as Brazil, South Africa, Tunisia and Australia, it is the most common cause of cardiac mortality in children [3, 17]. First attacks are uncommon in the very young (under the age of 5 years)[18] with the peak incidence occurring in those aged between five to fifteen with a decline thereafter and cases are rare in adults over the age of 35. Recurrent attacks are most frequent in adolescence and young adulthood and are diagnosed infrequently after the age of 45 years [2, 16]. As a result of its high prevalence in developing countries, RHD is the most common form of pediatric heart disease in the world. It is less prevalent in developed countries due to improved living conditions, but an increasing incidence has been reported in last 2 decades in developed countries [19].

A recent review of the global burden of GABHS-related disease estimated that there are at least 15.6 million people with Rheumatic Heart Disease (RHD), another 1.9 million with a history of RF but without carditis (who, however, still require preventive treatment), 470 000 new cases of RF each year, and over 230 000 deaths due to RHD annually[3]. Sub-Saharan Africa is most severely burdened by this disease with 5·7 cases per 1000. Within-country variation is evident in Australia, with the indigenous population accounting for 94% of all deaths due to RF [12].

CLINICALS ASPECTS

The description of symptoms most often associated with clinical RF made by T. Duckett Jones in 1944, improved the accuracy of diagnosis and helped to understand the disease. The important addition in 1965 of a requirement for evidences of a streptococcal infection refined these criteria. However, even with the framework of the Jones' criteria, diagnostic (and therapeutic) problems remain. Nevertheless, the recommendation by American Heart Association Expert Committee reaffirmed the usefulness of Jones' Criteria for diagnosing initial attacks of RF [2, 20, 21].

The clinical features of active RF comprise any one or a combination of the following: (i) carditis, (ii) polyarthritis, (iii) chorea, (iv) subcutaneous nodules, and (v) erythema marginatum. These constitute the major criteria of Jones. Minor criteria are: (i) an elevated ESR, (ii) positive C-reactive protein, (iii) raised white cell count, (iv) prolonged PR-interval on ECG (not valid if there is clinical carditis), (v) arthralgia (not valid if there is clinical arthritis), (vi) pyrexia, and a (vii) previous history of RF [2, 5, 22]. The clinical features that constitute the major criteria of Jones are discussed below.

Usually, the RF has an acute febrile onset and variables combinations of arthritis and arthralgia, carditis, chorea and skin manifestations [2]. Carditis is the only lesion in RF that may cause death or permanent sequelae. It is the most important factor in the disease determining prognosis, since all the others can be completely resolved without permanent ill-effects. Carditis is defined as acute, active, ongoing inflammation in cardiac tissue, be it pericardium, myocardium, or endocardium, whereas 'rheumatic heart disease' denotes the resultant chronic lesion remaining after the active disease [5]. These occur as a result of an autoimmune response against streptococcal antigens that develop cross-recognition of human cardiac tissue. The mimicry between streptococcal antigens and heart tissue proteins, combined with the production of pro-inflammatory cytokines and reduced production of interleukin 4, leads to the development of cardiac tissue

damage[23]. RHD affects the mitral valve in up to 50% of cases and results in mitral insufficiency, mitral stenosis, or both. In young patients, mitral regurgitation is predominant, and mitral stenosis becomes progressively more common with age [2, 15, 19].

As stated above, another clinical feature that constitutes one of the major criteria of Jones is chorea. Sydenham's, or rheumatic chorea manifests as repetitive, involuntary, jerky movements involving mainly the face and limbs. Hyperextension of joints because of severe hypotonia is seen, especially involving the metacarpophalangeal joints [5]. This is characteristically periodic. The larger joints: elbows, wrists, knees and ankles, are usually involved, with occasional involvement of smaller joints of the fingers. There is pain, tenderness, swelling and limitation of movement. When any of the other major criteria co-exist, the arthritis can be confidently diagnosed as being due to RF. However, difficulties arise when only one joint is involved with no other major signs, or if there is arthralgia without objective signs of arthritis [5, 15, 22].

The other clinical features are subcutaneous nodules (SN) and Bernier erythema marginatum (EM). The nodules of acute RF are never tender, and are most commonly found over the extensor surfaces of the elbows, knees, shins, and over the spine. EM is a transient rash in the form of wavy lines or erythematous rings with normal paler centres, occurring mostly on the trunk and proximal parts of the extremities but never the face, hands or feet [5, 15, 22].

GABHS PATOGENICITY

Almost every autoimmune disease that has been described can be linked to one or more specific infectious agents [24]. One of the best-recognized examples of this relationship is acute RF, which develops several weeks after infection with *Streptococcus pyogenes*. [25].

The pathogenic mechanisms involved in the development of RF are due to an abnormal humoral and cellular immune response [26]. The inflammation observed in RF presents a characteristic histopathological picture, including the presence of Aschoff bodies and leukocyte infiltration. Although the pathognomonic Aschoff bodies are rarely seen in the valves themselves, RF does lead to an acute valvulitis with inflammation and edema of the leaflets. Fibrin–platelet thrombi occur along the leaflet contact zones. Fibrosis of the affected valves leads to deformity, stenosis, and insufficiency. The progression to manifest RHD, particularly the calcification that accompanies RHD, was until recently thought to be a passive process attributable to the abnormal hemodynamics caused by the deformed valves [10, 11, 24, 25, 27]. However, it was later discovered that this response to the infection is related to the *Streptococcus pyogenes* strain. Studies of outbreaks of streptococcal pharyngitis show that certain M serotypes strains oare strongly associated with RF, whereas other equally prevalent strains do not initiate the disease or even reactivate it in highly susceptible hosts [15, 26]. The propensity of a given strain to elicit RF may depend on its virulent phase, on quantitative factors such as the reduction of M protein, hyaluronate, or other less well-defined biological properties. Virulence is likely to be enhanced in epidemiological settings that favour rapid person-to-person passage [10]. Moreover, variations in the rheumatogenicity of prevalent GABHS strains probably account for the marked temporal and geographic fluctuations in the incidence of RF [22].

Rheumatogenic streptococcal strains have distinct biological properties. Their M-protein molecules share a particular surface-exposed antigenic domain against which RF patients mount a strong IgG response. Surface structures of group A streptococcus include the family of M proteins, the hyaluronic acid capsule, and fibronectin-binding proteins that allow the organism to adhere to, colonize, and invade human skin and mucus membranes under varying environmental conditions. M protein binds to complement control factors and other host proteins to prevent activation of the alternate complement pathway and thus evade phagocytosis and killing by polymorphonuclear leucocytes. Extracellular toxins,

including superantigenic streptococal pyrogenic exotoxins, contribute to tissue invasion and initiate the “cytokine storm” which is believed to be responsible for illnesses such as necrotizing fasciitis and the highly lethal streptococcal toxic shock syndrome [10, 11, 22, 27, 28].

Indeed, the specific biological properties of the streptococcus causing pharyngitis allow the organism to (i) adhere to the pharyngeal mucosa and cause infection, and (ii) to resist phagocytosis. Although the exact mechanism by which GABHS induces the disease remains unexplained, much attention has been focused on the concept of autoimmunity, or, more precisely, molecular mimicry. This theory is rendered more credible by several examples of antigenic similarity between somatic constituents of GABHS and human tissues, including heart, synovium, and neurons of the basal ganglia of the brain. Taken together, these immunological cross-reactions could theoretically account for most of the manifestations of RF. As yet, however, there is no direct evidence that any of these manifestations are pathogenetically significant [10, 15, 28].

RECOMMENDATIONS FOR TREATMENT

There is no doubt that RF is a preventable illness and should receive high priority in primary health care programs in all developing countries. Although RF is theoretically preventable by antibiotic treatment of the streptococcal infections, once infection has occurred, there is no therapy proven to be able to prevent progression to valvular involvement apart from the prevention of recurrent episodes of RF [25, 29].

Antimicrobial therapy is vital, because without antibiotic treatment, some patients (1%) develop acute RF but, before the 1950s, the use of clinical cure as an efficacy endpoint in GABHS pharyngitis was unreliable. When it was proved that penicillin therapy could prevent acute rheumatic fever, it became clear that most cases of GABHS pharyngitis were self-limiting. Without antibiotic treatment, the tonsils and lymph nodes did not return to their normal volume for several weeks, but the great majority of patients showed resolution of their signs and symptoms within 7 days, and the patient's fever usually disappeared within 3 to 5 days. When it was observed that failure to eradicate GABHS was associated with the failure to reduce the incidence of the disease, a relationship was established between bacterial eradication of GABHS by penicillin therapy and the prevention of acute RF. Therefore, trials comparing the clinical efficacy of various antibiotic regimens use the endpoint of bacteriologic eradication of GABHS as a surrogate marker of prevention. This is reflected in the guidelines of Food and Drug Administration and European Medicines Evaluation Agency for the development of new antibiotics for GABHS pharyngitis, which both use bacteriological eradication of the initial pathogens as the main efficacy endpoint [28, 30, 31].

In 1950, it was reported that acute RF could be prevented by the treatment of streptococcal pharyngitis with penicillin. The following year, a new repository form of penicillin, benzathine penicillin G (BPG), was studied and reported to be effective in secondary prophylaxis for prevention of recurrent RF [32]. Both the World Health Organization and the American Heart Association recommend injections every 3 weeks of

1200 000 U BPG for secondary RF prophylaxis in certain high-risk patients or in highly endemic areas. Official recommendations usually cover two phases: treatment of acute pharyngitis attacks and prophylaxis, as discussed below.

The treatment of acute streptococcal pharyngitisis based on bed rest until symptoms have subsided and doses of penicillin sufficient to maintain bactericidal blood levels for 10 days in order to eradicate the infecting streptococci. Short courses of salicylates can be used to obtain symptomatic relief from arthritic pain, but have not been shown to be of value in carditis. Corticosteroids are recommended by the some practitioners but they have not proved to be effective in the management of carditis: they neither shorten the duration of the acute attack nor have any beneficial effect on the long-term outcome. In patients with heart failure, digoxin, diuretics with potassium supplementation, and occasionally, captopril, may be indicated [3, 5, 15].

Prophylaxis can be divided into primary and secondary. Primary prophylaxis refers to antibiotic treatment of Group A streptococcal pharyngitis to prevent subsequent RF [8]. A single intramuscular injection of 1.2 million units of benzathine penicillin or 10 days of penicillin V is advised but in communities with a high prevalence of RF a intramuscular injection of BPG is usually the first treatment choice, because of poor compliance with 10-day regimens of oral penicillin [22]. Efforts at primary prevention are hampered by the fact that many patients who develop RF are not aware of a preceding sore throat. There are no simple specific clinical signs to diagnose streptococcal pharyngitis, and throat swabs and cultures are expensive. However, antibiotic treatment for suspected streptococcal sore throat is effective in reducing the occurrence of subsequent attacks of RF in 70–80% of the cases, and this may be affordable for developing countries as a strategy for preventing RF [3].

Secondary prophylaxis, the long-term administration of antibiotics to prevent recurrence, is of proven benefit and cost-effective [2]. Benzathine penicillin at 1.2 million units, intramuscularly, every four weeks, is the standard recommendation. Secondary

prophylaxis has been advised until age 21 or at least 5 years after the last attack of RF, whichever is the longer. The WHO points out that it is not possible to generalize, and the duration of secondary prophylaxis should be individualized taking into account factors with influence the risk of recurrence. Lifelong prophylaxis is recommended for patients with severe valve disease or after valve replacement surgery [2].

For such long-term prophylaxis, twice- or thrice-weekly injections (or injections every two or three weeks?) are more effective but may be more difficult to implement. Penicillin V 250 mg orally twice daily is recognized to be less effective but may be preferred by some practitioners, particularly in very thin patients who are on warfarin anticoagulation after valve replacement surgery when deep intramuscular injections may be undesirable. The drawbacks and limitations of the penicillin V 10-day regimen have led to the study of other antibiotic compounds for the treatment of GABHS pharyngitis [28].

For almost five decades, penicillin has been the drug of choice for the treatment of Streptococcal pharyngitis [33]. This antibiotic has proven efficacy and safety with a narrow spectrum of activity, as well as low cost. However, some other drugs have also been employed. For example, cephalosporins have been successfully used for the treatment of GABHS tonsillopharyngitis since the early 1970s [34]. Several antibiotics (amoxicillin, cefadroxil, cefuroxime, cefpodoxime, cefixime, cefotiam, cefdinir, azithromycin, clarithromycin), with simpler and shorter dosing regimens provide similar or better results than 10-day penicillin V in terms of GABHS eradication. Azithromycin is the most extensively studied drug in the setting of pediatric GABHS pharyngitis, probably because of its pharmacokinetic properties [28, 30, 35].

In addition to the regimens consisting in the administration of penicillin or cephalosporins, another therapeutic approach involving a slight physico-chemical modification of penicillin resulted in the introduction of BPG some decades ago [35]. This provides a depot form of the antibiotic, which has now become the drug of choice for

prevention of RF. Pharmacokinetic studies indicated that serum penicillin levels exceeded the minimal inhibitory concentrations of Group A streptococci for 4 weeks after intramuscular injection of 1 200 000 units [36]. The minimum inhibitory concentration (MIC) of penicillin reported against GABHS is estimated to be between 0.01-0.03 µg/mL [35]. A level of 25 ng/mL was chosen to indicate an adequate protective penicillin level and there was a consistent but not a statistically significant trend of higher proportions of patients with plasma penicillin levels above 25 ng/mL with as the BPG dose given each week was increased. The choice of 25 ng/mL as a protective level of serum or plasma penicillin is based on published penicillin MICs for *Streptococcus pyogenes*, while the vast majority of organisms have MICs below 20 ng/mL. One study found over 80% of samples from 21 days after 1 200 000 U BPG had penicillin levels greater than 20 ng/mL [32, 35].

Further pharmacokinetic studies reported that intra-muscular injection of BPG did not produce serum values above the MIC by week 3 in a significant proportion of patients [36] . Data challenging the 4-week schedule have been published. It was reported that very low or non-detectable values 4 weeks were obtained after administration of 1 200 000 units of benzathine penicillin G, and that injections given at 3-week intervals resulted in fewer recurrences than injections given at 4-week intervals. The recurrence rates were 3.0% and 9.7% respectively. More recent studies reported that intramuscular injection of BPG did not produce serum values above the minimal inhibitory concentration by week 3 in a significant proportion of patients [36].

The penicillin concentrations in tonsils after the administration of BPG have also been investigated. They indicate that consistent concentrations were maintained for 2 weeks after administration followed by a significant reduction in the values at day 21. These findings provide a further explanation of why recurrences of rheumatic fever are observed in patients treated with the monthly schedule for prophylaxis. These results indicate that penicillin concentrations in sera and tonsils may be inadequate for prevention of rheumatic fever by

week 3 of administration in most children after administration of 40 000 IU/kg benzathine penicillin G [36, 37].

Several reports have recently appeared in the literature questioning the persistence of effective penicillin levels beyond the third week after the BPG injection [38]. The recommendation of the American Heart Association of monthly injections is now being questioned by physicians in developing countries. In India, a prophylaxis regimen based on injections administered every 3 weeks had been used during the 1980s. In Brazil, two modified regimens have been proposed. One of them consists of the regular injection every 2 weeks for all ages. Furthermore, some authors have suggested that a logical approach for secondary prevention would be a 3-week program in areas where the risk of RF recurrences is high [38, 39].

Other studies have focused on the pharmacokinetic profiles obtained from BPG preparations of different suppliers. Although the contents of the two products analyzed were found to be chemically equivalent (1.2 million U), a remarkable difference between them was observed. This difference might be due to the physical properties of benzathine penicillin, the degree of solubility, and hence, the rate of release of penicillin from the site of injection [39].

As well as the influence of the physical properties of, BPG, the limited success in preventing streptococcal infections might be due to several interacting factors: including the compliance of patients, the chronically inflamed tonsillar tissue that hinders the penetration of penicillin and the coexistence of GABHS with other penicillinase-producing organisms. Further investigations are needed to elucidate this question [39, 40].

Another explanation could lie in interactions between GABHS and other members of the pharyngo-tonsillar bacterial flora. For example, it is hypothesized that beta-lactamase secreted by betalactamase-producing bacteria, which colonize the pharynx and tonsils, may ‘‘shield’’ GABHS from penicillin. Another possibility is aggregation between *Moraxella catarrhalis* and GABHS, which could facilitate colonization by GABHS. Normal bacterial flora

can interfere with the growth of GABHS, and the absence of such competitive bacteria makes it easier for GABHS to colonize and invade the pharyngo-tonsillar area [41].

The reasons for treatment failure are multifactorial and include difficulties with health service delivery and inadequate numbers of health staff in addition to a mobile population and different cultural perceptions of health and sickness. Under such circumstances, it was considered that a BPG regimen of one injection every 3 weeks would generally be unacceptable and impractical [32].These data suggest that doses larger than the currently recommended ones of benzathine penicillin G may be more appropriate for prevention of RF, particularly in higher risk populations. Although early studies of BPG suggested that larger doses resulted in prolongation of the period of detectable penicillin levels, there are inadequate data on secondary prophylaxis BPG regimens with doses greater than the 1 200 000 U [32]. Another possibility is to consider more frequent injections, although this may carry an increased risk of poorer compliance [36].

Indeed, compliance depends on the length of treatment, with evidence for better compliance with shorter regimens. Drug palatability may also be one of the most important factors involved. Patient compliance has a major impact on the efficiency and safety of a medication, and it is an important variable to consider when selecting an antimicrobial agent.

NANOCARRIERS

As mentioned above, the poor penetration of penicillin into the tonsillar tissues and tonsillar surface fluid, and microbiological interactions between GABHS and other pharyngo-tonsillar flora can account for the failure of penicillin to eradicate GABHS [41]. In order to overcome these difficulties, innovative approaches are required.

During the last three decades, colloidal vehicles have been explored and have emerged as prospective systems for drug delivery. These supramolecular systems often lead to improvement in the therapeutic index of the lipophilic drugs through increased solubilization and modification of their pharmacokinetic profiles. The potential applications of colloidal drug carriers by the intravenous route can be summarized in terms of the (i) concentrating drugs in sites accessible to the carrier; (ii) rerouting of drugs away from sites of toxicity; and (iii) increasing the circulation time of labile or rapidly eliminated drugs. Carriers are able to leave the circulatory stream and pass into inflamed or infected sites, where the capillary endothelium is defective. Some formulations based on colloidal drugs carriers are already on the market and are able to reduce the side effects and control the release of the encapsulated drugs. Colloidal drug carriers are particularly useful for drugs produce”d by biotechnology (proteins and nucleic acids) because they can provide protection from degradation in biological fluids and promote their penetration into cells. They are also useful for the formulation of small hydrophobic molecules, because they can provide an ultradispersed form without the use of irritating solvents and allow rapid drug dissolution. Therefore, it is likely that colloidal systems able to improve the efficacy of both established drugs and new molecules will soon be available [42].

Microemulsions, an example of colloidal carriers, are spontaneously forming single phase colloidal dispersions of either oil-in-water or water-in-oil stabilized by an interfacial film of surfactants and cosurfactants. These self-assembled dispersions have low viscosity, very

low interfacial tension, good shelf-life, high solubilizing capacity, macroscopic homogeneity, and microscopic heterogeneity (microdomains) [43].

Considering all these advantages, the use of microemulsions for different fields has been reported in the literature [44]. The focus in this article is the potential applications of microemulsions in the pharmaceutical area, and more precisely for improving the treatment of RF. While it is true that an increase in solubility of drugs in microemulsion system is an important factor in their performance, another important factor is their small droplet size, resulting in large surface area from which the drug can partition and be absorbed or permeate through membranes, and the dissolution route is no longer limiting. The encapsulation of drugs in the microdomains also offers protection from enzymatic degradation by the interfacial layer. Furthermore, the membrane permeability is facilitated due to the presence of surfactants and cosurfactants [45].

Other potential advantages of microemulsions include their transparency. This property enables microemulsions to be visually assessed for microorganism growth and also allows inspection for the presence of undissolved drug. The thermodynamic stability is also an important characteristic of these systems when compared with kinetically stabilized macroemulsions. In addition, the formation of microemulsions requires only the most basic mixing equipment. More importantly, their manufacture is not as dependent on the careful control of manufacturing process as, for example, during the preparation of macroemulsions [45, 46].

The increase in bioavailability for drugs included in microemulsions compared to more conventional dosage forms have also been discussed[43]. Several researchers have reported studies in which a reservoir of the drug is produced and sustained release effect is achieved. Drug release from a microemulsion formulation depends on several factors, knowledge of which is important to a formulation scientist. In these studies, the drug release rate was controlled by its partitioning from the oil to the water phase of the system. Other

factors such as phase volume ratio, droplet size of the dispersed phase, distribution of the drug in the various phases of the system, potential interaction between the additivess and drug, and the rate of drug diffusion in both phases of the system may also play an important role in the drug release. A clear understanding of these important parameters and a careful selection of all the additives are essential for optimizing drug release from the microemulsion system.

Considering all the points mentioned above, microemulsions may be very attractive vehicles for the parenteral administration of penicillin. They may enable a sustained release and thereby reduce the frequency of administration. Patient safety would also be improved because a reduced dose would be required and the plasma concentration would be stable. However, very few studies in the literature have addressed the use of nanocarriers for RF treatment. One published work reports that Penicillin G can be incorporated into liposomes [47], and although these systems remained stable for over a year and showed in vitro activity against *Streptococcus pyogenes* , these liposomes were not able to encapsulate a sufficiently large amount of penicillin. Nanoemulsion and nanocapsules were also used to formulate colloidal nanosystems of the BPG intended for the prophylactic treatment of RF [48]. However, the penicillin concentration in nanocapsules was about 100 times less than the therapeutically recommended dose for children. Development of polybutyl adipate (PBA) nanocapsules loaded with penicillin-G has been reported[49]. The drug was successively encapsulated within PBA nanocapsules with high drug loading and encapsulation efficiency using the w/o/w emulsion solvent evaporation technique, but these nanocapsules showed high burst release. Others systems, such as a micellar system of BPG, have also been reported [50]. This micellar system could incorporate to 90% incorporation of BPG, despite the incompatibility of this molecule with water, and improve its resistance against the hydrolytic and enzymatic degradation., The systems described so far indicate that there is potential in the development of colloidal drug carriers for BPG, however, further work is still

needed to reach a new and therapeutically appropriate system for BPG which could improve the conditions of treatment of patients with rheumatic fever.

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BENZATHINE PENICILLINE G: ASPECTS CHIMIQUE, PHARMACOLOGIQUES ET ANALYTIQUES.

Les pénicillines forment la classe des antibiotiques β-lactamines, produite à partir de cultures des champignons *Penicillium* tels que *P. notatum* ou *P. chrysogenum* (16, 17). La pénicilline G (PenG) est une pénicilline naturelle qui a été obtenu grâce à la découverte par Alexander Fleming en 1928, après l'isolement d'une substance qui inhibait les staphylocoques dans une plaque de gélose contaminés par *Penicillium* et le succès de Howard Forey, Ernst Chain, en 1942, dans le procédé d'extraction de cette substance, appelée la pénicilline par Fleming (16-19).

La structure de base des pénicillines comprend au noyau de l'acide 6-aminopenicillanique (6-APA) et une chaîne latérale (20). Le 6-APA est issu de la réaction entre deux acides aminés: la cystéine et la valine ; lorsque ces deux structures sont ajoutées, il y a la formation du noyau bicyclique de la pénicilline. Ce noyau est responsable de l'activité biologique de la molécule et se compose d'un anneau thiazolidine, relié à un anneau β-lactamines. Le type et la taille de la chaîne latérale détermine les propriétés antibactériennes et pharmacologiques. La PenG, par exemple, est formée par la réaction de l'acide phénylacétique de la 6-APA à le radicale benzyle (chaîne latérale). Pour modifier les propriétés physico-chimiques de cette molécule des radicales amines comme la procaïne et benzathine peut être incorporées dans sa structure, pour former des sels peu solubles (19, 21)

Des nombreuses formulations des sels de PenG sont disponibles. Parmi ceux-ci on peut citer les sels de sodium et de potassium qui sont soluble dans l'eau et sont disponibles dans les formes de comprimés, gélules et poudres pour solution injectable. Les moins solubles, sels de procaïne et benzathine, ne sont disponibles qu'en formulations pour l'injection de longue durée. Comme la principale incompatibilité de PenG est avec de l'eau, les comprimés doivent être conservés à l'abri de la humidité, les poudres à sec et l'injection contient généralement des agents tampons qui maintiennent le pH environ 6, proche de la valeur appropriée (11). L'agrégation au site d'injection après l'administration est suggéré par

certains auteurs d'être une conséquence des propriétés colloïdale de la PenG. Selon eux, l'autoagrégation de la PenG se produit en deux étapes: en première la formation de dimères de la molècule, suivie par la formation de micelles si la concentration micellaire critique est atteinte (22).

1.1.1 Propriétés physico-chimiques

La pénicilline G benzathine (PenGB) est produit par réaction de deux molécules: PenG et dibenzylethylenediamine, et est chimiquement connu comme l'acide (2S, 5R, 6R)-3.3-diméthyl-7-oxo-6-(2-phénylacetamide)-4-tia-1-bicyclo [3.2.0] heptane l'acide-2-carboxylique combinée avec la N, N'-tétrahydraté dibenzyle éthylène diamine (2:1) (21). Comme toutes les pénicillines, la PenGB est optiquement active et due au groupe carboxyle, elle est une acide relativement forte avec un pKa autour de 2,65 (21, 23). La structure chimique est illustrée dans la Figure 1.

Cette molécule se presente comme un poudre cristalline blanche inodore, très peu soluble dans l'eau, modérément soluble dans l'alcool et pratiquement insoluble dans le chloroforme et l'éther. Elle est thermosensible et devraient être entreposée dans des contenants étanches à l'air à des températures inférieures à 30°C. Il a un point de fusion entre 214 et 217 °C, et caractéristique lipophilie avec coefficient de partage (log P) égal à 2,1979 (21, 24-26).

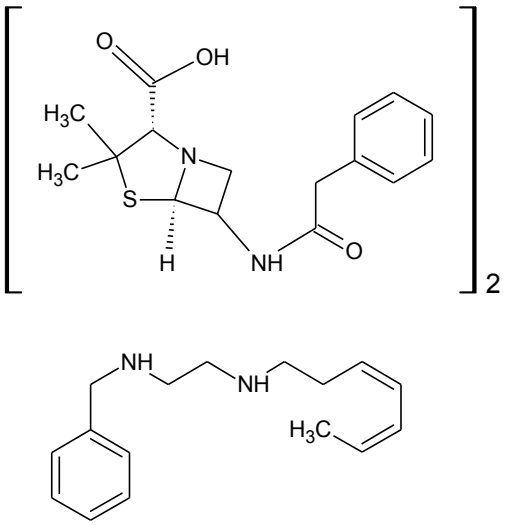

Figura 1. La structure chimique de la PenGB

A cause du tension exercée sur la liaison amide dans le cycle β -lactamines, la PenGB, comme tous les autres molécules de sa catégorie, est très réactif, ce qui lui rend sensible aux attaques nucléophiles et électrophiles, qui en font une molécule que se dégrade facilement soit par hydrolyse en milieu aqueux, soit suite à une activité enzymatique (27, 28). Quand l'hydrolyse se produit dans des conditions alcalines, les acides pénicilliniques sont formées par simple rupture de l'anneau β -lactamine. En milieu neutre, le processus est similaire, bien que plus lente ; quand le pH et la température sont maintenus constants, la réaction suit une cinétique de premier ordre. La dégradation enzymatique par l'amidase conduit à la 6-APA et un grouopement carboxylique à partir d'une rupture de la molécule en position 6-amino de la chaîne latérale. Par action de β -lactamases, qui provoquent une rupture dans le cycle β-lactamine, parmi les produits de dégradation, l'acide penicillinque a été identifié dans l'hydrolyse en milieu alcalin (27). Comme toutes les pénicillines, la PenGB peut réagir avec les alcools et les amines pour former des esters et des amides correspondants (11, 20, 29).

L'action antimicrobienne de la PenGB est similaire à celle de PenG et à cause de faibles concentrations de cette molécule dans le plasma, son utilisation est limitée aux micro-organismes très sensibles à PenG. Elle a une action bactéricide principalement contre les bactéries Gram positives, certaines cocci Gram-négatives, les spirochètes et des actinomycètes. Elle est utilisé pour traiter la syphilis, la gonorrhée, les infections à streptocoques et Treponema, en particulier celles qui affectent les voies respiratoires et pour la prévention des récidives de rhumatisme articulaire aigu (26).

La sensibilité des bactéries à la pénicilline est très variable, même entre les classes normalement sensibles, surtout lorsqu'il ya des incidences des organismes produisant β -lactamases. La production de ces enzymes est le mécanisme le plus important de la résistance développée par les bactéries. Ces enzymes sont produites par des

staphylocoques et des bactéries Gram négatif, et son, action est principalement la destruction de β-lactame désactivant ainsi la pénicilline (30).

L'action bactéricide de la BPG est due à l'interférence causée dans la phase finale de synthèse de la paroi cellulaire des bactéries, le médicament se lie à des protéines spécifiques, appelées protéines de liaison à la pénicilline (PLP) et produit des changements morphologiques comme la formation de longs filaments ou des anomalies de la paroi cellulaire. Les pénicillines inhibent la transpeptidase, qui est responsable de la réticulation entre les différents chaînes de peptidoglycane. Cette inhibition est due à liaison covalente avec un résidu sérine dans le site actif de l'enzyme. Il y a la formation du complexe pénicilline-enzyme qui ne peut pas être hydrolysé. Ainsi, la transpeptidase est irréversiblement inhibée par le médicament bloquant la dernière étape de la biosynthèse de la paroi cellulaire bactérienne, causant ainsi la mort de cellules (18, 19).

La PenGB est stable en présence de jus gastrique, mais son absorption dans le tractus gastro-intestinal est variable et la fraction absorbée est très faible (26). Ainsi, la forme habituelle d'administration de la PenGB actuellement est une suspension pour injection intramusculaire. Après administration, il y a la formation d'un dépôt d'où le médicament est libéré lentement et hydrolysé pour donner de la PenG. La concentration plasmatique maximale est atteinte en 24 heures et une concentration efficace (20ng/mL) est maintenue pendant environ 4 semaines. A cause de la libération promongée, la PenG peut être détectée dans l'urine pendant les 12 heures suivant l'administration. L'activité spécifique de la pénicilline est définie en unités internationales (UI) : dans le cas de PenGB, 900 mg du médicament pur équivaut à environ 1.200.000 UI (26).

Depuis la découverte de la pénicilline, plusieurs techniques ont été développées pour son analyse, y compris les tests microbiologiques et les dosages colorimétriques, chromatographiques et spectroscopiques (33). Le PenGB est cité dans plusieurs pharmacopées (25, 34, 35) et des techniques d'analyse sont mentionnés pour l'identification

et le dosage . Ces dernières années, un nombre considérable de méthodes très sensibles ont été proposés pour l'analyse de ce médicament dans différentes milieux comme le lait, la nourriture, des échantillons de tissus et des formulations pharmaceutiques (36-42).

Les méthodes couramment utilisées pour l'analyse quantitative des antibiotiques en général sont les méthodes de chromatographie, de spectrophotométrie, d'analyse microbiologique et de radio-immunologique. Les méthodes microbiologiques prennent en compte le médicament ainsi que ses métabolites actives, ce qui est nécessaire pour les essais de bioéquivalence et la biodisponibilité dans les lignes directrices de l'Union européenne et la FDA. Cependant, ces méthodes utilisent des micro-organismes qui sont très sensibles à certains médicaments et dans de nombreux cas ils souffrent d'un manque leur spécificité par rapport aux méthodes chromatographiques en particulier en chromatographie en phase gazeuse (GC) et chromatographie en phase liquide (HPLC). Des dosages immunologiques ont été proposées récemment dans le cadre des essais cliniques et biomédicales, mais ces méthodes ne sonrt pas encore applicables à l'analyse chimique de routine (42).

Les méthodes de chromatographie en phase gazeuse sont rapides et précis, mais ils nécessitent des températures de fonctionnement élevées qui peuvent causer une dégradation thermique du médicament, puis ont souvent besoin d'une étape avnat la mesure pour accroître la volatilité et d'améliorer le comportement chromatographique. La chromatographie sur couche mince (CCM) et sa modification récente la chromatographie sur couche mince à haute efficacité (CCMA) sont utilisés dans divers domaines de l'analyse des antibiotiques, mais les résultats sont moins précis que ceux obtenus par HPLC (42). L'HPLC est une méthode particulièrement importante pour l'isolement et la purification d'antibiotiques. Sa capacité d'analyser des combinaisons des molécules volatiles et non volatiles et de déterminer les concentrations minimales durant un processus de séparation, fait d'elle une méthode de choix dans les laboratoires cliniques (42, 43). Dans certains cas, il est nécessaire de complémenter l'HPLC avec une autre technique d'analyse, telle que la

spectrométrie de masse (MS) (39), ou n'importe quelle étape précédente comme dérivation, la concentration ou la séparation de l'échantillon dans un pré-colonne (41, 43, 44) ce qui rend cette technique très coûteuse et difficile à mettre en œuvre.

Malgré sa relative simplicité, la spectrophotométrie est également proposée par les recueils de la pharmacopée (25, 34) et a été utilisé pour analyser PenGB dans des formulations pharmaceutiques avec de bons résultats (38, 45). Les principales caractéristiques de cette méthode sont une large applicabilité, une haute sensibilité, une bonne sélectivité, la précision, la souplesse, la facilité et le coût relativement faible. Ces facteurs font d'elle la technique de choix dans de nombreux laboratoires pharmaceutiques et les produits chimiques dans l'analyse de routine (32). Dans cette classe de la méthode analytique, la spectrophotométrie d'absorption atomique est particulièrement populaire en raison de la facilité du processus.

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MICROEMULSOES E NANOEMULSOES: ASPECTOS TEÓRICOS E TECNOLÓGICOS.

Devido ao grande aumento no número de sistemas carreadores para moléculas insolúveis em água, em fase de ensaio clínico, os sistemas baseados em lipídios tem sido uma boa opção para administração dessas moléculas, especialmente para modular e ou aumentar absorção daquelas que apresentam taxa de dissolução limitada[1]. No caso de fármacos pouco solúveis em água a taxa de dissolução pode ser extremamente baixa em condições fisiológicas conduzindo a uma biodisponibilidade inadequada.

Várias abordagens podem ser feitas no que concerne à formulação de um sistema de liberação para fármacos insolúveis. Se o composto tem grupos ionizáveis, a formação de sal é freqüentemente a primeira estratégia utilizada para melhorar a solubilidade. Caso contrário, o uso de cosolventes talvez seja a alternativa mais comum, provavelmente devido à alta eficiência e facilidade de uso dos cosolventes para solubilizar fármacos. No entanto, o uso de cosolventes tem o potencial de causar problemas como: precipitação do fármaco durante a diluição, dor e/ou danos no tecido durante a administração parenteral. Outras abordagens para incremento da solubilidade de fármacos incluem: desenvolvimento de pró-farmacos, sistemas micelares de surfactantes e/ou polímeros, complexos com lipídeos ou ciclodextrinas e lipossomos. O uso de lipossomas e ciclodextrinas têm se mostrado resultados promissores com alguns fármacos insolúveis em água, embora a capacidade da membrana de lipossomas e da cavidade interna das moléculas de ciclodextrina seja bastante limitada [2-6].

Em se tratando de formulações baseadas em lipídeos, os sistemas emulsionados são os mais comuns para administração de medicamentos. Ao longo das últimas décadas, as formulações de emulsões têm sido exploradas para a resolução de uma série de desafios na liberação de fármacos, pois ao contrário das soluções para a administração oral e parenteral, que geralmente são sistemas homogêneos uma fase molecular ou dispersões, emulsões são dispersões coloidais de pelo menos duas fases imiscíveis estabilizadas com o auxílio de um terceiro componente, geralmente referido como agente emulsificante,

surfactante ou tensoativo. As vantagens das emulsões em relação às formas convencionais de soluções orais ou injetáveis podem ser atribuídas a essa heterogeneidade estável e a capacidade de liberar fases imiscíveis de uma maneira confiável e reprodutíve[1, 7]l. Além disso, abordagens combinadas de emulsões com outras estratégias de formulação também pode ser desenvolvidas. Os cosolventes, por exemplo, são freqüentemente usados para incorporar fármacos em uma emulsão. Da mesma forma, a utilização de complexos com ciclodextrinas ou surfactantes para aumentar a afinidade do fármaco com uma das fases da emulsão, pode ser considerado uma abordagem que combina a formação de complexos e a estratégia de emulsificação[8].

O conceito de emulsão no *lato sensu* é conhecido desde a Grécia antiga quando Galeno desenvolveu o Cold Cream. Na segunda metade do século XXI, esse conceito evoluiu significativamente devido ao avanço nas áreas de tecnologia química e farmacêutica, e também devido às novas necessidades criadas pelo mercado. Nesse contexto, as emulsões galênicas, de simples misturas ordinárias de água e óleo estabilizadas por um tensoativo derivaram para o grupo que podemos intitular de “sistemas emulsionados”, onde estão inclusos todos os sistemas formados à partir de uma mistura de água, óleo e tensoativo em um equilíbrio particular, e dentre os quais, podemos citar: as emulsões ordinárias ou macroemulsões; as emulsões submicrônicas, também conhecidas também como nanoemulsoes; as microemulsoes, os sistemas liquido-cristalinos e ainda, os sistemas classificados como autoemulsionantes. A principal vantagem desses sistemas é o potencial de aumentar a solubilidade e com isso a biodisponibilidade dos fármacos[9-13].

Recentemente o interesse nas emulsões coloidais tem aumentado, e vários desses sistemas têm sido examinados e explorados, como veículos para a liberação controlada de fármacos. O uso do termo "microemulsão" na literatura cientifica de colóides e surfctantes compreensivelmente causa confusão, sobretudo entre cientistas farmacêuticos. Intuitivamente, pode-se supor que este termo significa uma emulsão ultrafina, compreendendo gotas na faixa submicrométrica. Como emulsões são termodinamicamente

instáveis, tendendo sempre para reduzir a área total da interface óleo-água, pode-se supor que as microemulsões sejam também termodinamicamente instáveis. No entanto, esse termo tem sido amplamente utilizado para descrever sistemas complexos geralmente compostos de óleo, surfactantes, cosolventes e água, que são termodinamicamente estáveis. Muitas vezes, a morfologia exata dessas misturas complexas não é totalmente compreendida, devido ao papel de cosolventes. Freqüentemente, as fases de óleo e água não podem ser fisicamente definidas, de modo que não é possível estabelecer qual é a fase contínua, e isso tem causado divergências na literatura farmacêutica quanto ao uso desse termo [14-16].

A alta estabilidade cinética, baixa viscosidade e transparência óptica tornam tanto as microemulsoes como as nanomemulsoes muito atrativas para muitas aplicações de sistemas industriais, por exemplo, no campo farmacêutico como sistemas de liberação de medicamentos, em formulações de cosméticos, cuidados pessoais, em agrotóxicos para a liberaçao de pesticidas, e na indústria química como meio de reação de polimerização. O desses sistemas como carreadores coloidais de fármacos é bem documentada [12, 17-20].

O objetivo desse capítulo é discorrer sobre as propriedades dos sistemas emulsionados em escala nanométrica e principalmente diferenciar do ponto de vista teórico e tecnológico as nanoemulsoes e as microemulsoes.

NANOSISTEMAS EMULSIONADOS

Como citado anteriormente, no rol dos sistemas emulsionados, encontram-se atualmente em grande evidencia, àqueles em escala nanométrica como as microemulsoes e nanoemulsoes, que devido à alta capacidade de solubilização, enorme área interfacial, a grande estabilidade são de grande interesse para a indústria química e farmacêutica.

O conceito de microemulsão foi introduzido em 1943 por Hoar & Schulman quando conseguiram gerar uma solução monofásica clara, titulando uma emulsão leitosa com hexanol[21]. Em 1981, Danielsson & Lindman propuseram uma definição oficial para microemulsão: "microemulsão é definida como um sistema de água, óleo e tensoativo formando uma única solução opticamente isotrópica e termodinamicamente estável"[13]. De acordo com Winsor, esses sistemas podem ser classificados no diagrama de fases e essa classificação depende basicamente da natureza e do número de fases líquidas presentes. (a) Winsor I, contendo uma fase oleosa em equilíbrio com uma fase emulsionada, (b). Winsor II, também bifásica, constituída de uma fase aquosa em equilíbrio com uma emulsão, (c) Winsor III, trifásica, contendo uma fase aquosa e outra oleosa, separadas por uma fase emulsionada, e (d) Winsor IV, correspondente a uma região monofásica, representada por uma emulsão homogênea (Figura 1)[22].

I II III IV

Figura 1. Representaçao esquemática da classifocaçao de Winsor.

As microemulsões são transparentes e/ou translúcidas por causa do tamanho das gotas da fase dispersa (<200nm). Algumas das vantagens associadas às microemulsões como sistemas de liberação de fármaco são: incremento da solubilidade e

biodisponibilidade, proteção contra a hidrólise enzimática, possibilidade de uso em vetorização de fármacos, além de todas essas vantagens farmacológicas, existem também àquelas tecnológicas como: estabilidade termodinâmica, facilidade na preparação e transposição de escala[1, 6, 14, 16, 18, 23].

Esses sistemas possuem uma tensão interfacial muito baixa e a energia de superfície das gotículas dispersas é grande. A baixa tensão interfacial compensa a entropia do sistema e por isso esse tipo de dispersão é considerado termodinamicamente estável, o que torna mais interessantes frente aos outros sistemas emulsionados. Para a diminuição da tensão interfacial necessária para a formação de uma microemulsao é necessário a adição de um segundo agente de superfície, normalmente denominado co- surfactante. Obviamente, diferem das macroemulsões (ou emulsões simples), que têm estabilidade limitada e maior tamanho de gotículas, não são transparentes, e exigem uma quantidade importante de energia para serem formadas. As microemulsões diferem também das micelas reversas. Mesmo as microemulsões de água em óleo que são consideradas semelhantes topologicamente às micelas reversas, devido à orientação das cabeças polares do surfactante, diferenciam-se devido à presença de água livre no centro da gotícula, enquanto as micelas reversas apresentam um núcleo rígido, devido à imobilização de toda a água presente para hidratar as cabeças polares do anfifílico e contraíons . Outra diferença está no tamanho, que, para micelas reversas é normalmente restrito a 5 nm, enquanto as gotículas de microemulsões são geralmente maiores, também devido à presença de uma certa quantidade de água livre[16, 22].

As nanoemulsões, que também são referidas na literatura como miniemulsões, emulsões submicrométricas ou emulsões ultrafinas, e que não obstante são confundidas com microemulsoes, são emulsões (sistemas não-equilíbrio), com um tamanho de gotícula em escala nanométrica, por exemplo, 20-200nm, apesar de alguns autores considerarem 600 nm como o limite superior. E que devido ao seu tamanho característico, se apresentam também transparentes ou translúcidas a olho nu, assim como as microemulsoes, e possuem

estabilidade contra a sedimentação ou cremagem. Mas, ao contrário microemulsões, nanoemulsões são apenas cineticamente estáveis[17, 24].

O tamanho das gotículas de nanoemulsões torna-a resistente à desestabilização física, através da separação gravitacional, floculação e/ou coalescência (Figura 2). Esses sistemas são resistentes à cremagem porque seu movimento browniano é o suficientemente baixo para superar a sua força de separação gravitacional. Eles também são resistentes a floculação devido à eficiência da estabilização estérica. A maioria das nanoemulsões é estabilizada por surfactantes sintéticos, que tendem a ter longas caudas hidrofílicas da ordem de 20-10 nm. No entanto, as nanoemulsões são particularmente propensas a um crescimento no tamanho das gotículas ao longo do tempo por um processo conhecido como maturação de Ostwald ou de difusão molecular. Esse fenômeno é o principal mecanismo de desestabilização desses sistemas e é dependente das características de polidispersividade do sistema. Neste processo, as gotas de tamanho maior crescem em detrimento as gotículas menores, devido à difusão molecular da fase dispersa através da fase contínua. Assim, é considerado que as nanoemulsões são cineticamente estáveis, mas termodinamicamente instáveis. E essa, é a principal diferença em relação às microemulsoes[3, 25, 26].

COMPOSIÇAO

Em termos de composição as microemulsoes se assemelham bastante às nanoemulsoes, no entanto à proporção entre as fases e a concentração de tensaotivos varia consideravelmente entres esses sistemas. Numa formulação de microemulsao é necessário uma quantidade de tensoativo maior que numa formulação de nanoemulsao utilizando os mesmos componentes[3, 8]. As microemulsões diferem das nanoemulsões não somente por serem opticamente transparentes e isotrópicas, ou ainda pela estabilidade termodinâmica, mas também por apresentarem diferentes apresentaçoes em escala nanométrica, como estruturas bicontinuas, esféricas, tubulares ou lamelares, o comportamento dessas

estruturas está relacionado com a mudança de alguma variável termodinâmica como a temperatura, composição e diluição[15].

Tanto as nanoemulsoes quanto as microemulsões podem ser "sistemas pseudo-ternários" (onde o tensoativo e cotensoativo estão juntos formando uma fase única). As dispersões são formadas quando o óleo, água e tensoativo/cotensoativo são misturados em proporções adequadas. O comprimento de cadeia do co-tensoativo varia de C2 e C10 e a natureza anfifílica desses agentes faz com que esses interajam com as monocamadas de surfactante na interface entre os líquidos afetando assim a sua organização. Fases líquido-cristalinas são formadas quando o filme surfactante é muito rígido. Os cosurfactantes penetram na monocamada de surfactante, proporcionando fluidez adicional para o filme interfacial e, consequentemente, interrompendo a formação de fases líquido-cristalinas. Além disso, os cosurfactantes também distribuem-se entre a fase aquosa e oleosa, alterando assim a composição química da mistura e, portanto, o HLB do sistema[15, 25, 27].

A formação das nanoemulsoes e microemulsoes dependerá dos seguintes fatores: (I) O HLB do surfactante determina o tipo de sistema através de sua influência no empacotamento molecular e a curvatura do filme. (II) Propriedade do surfactante, a fase oleosa e a temperatura: O tipo de sistema depende da natureza do surfactante. Quando uma alta concentração do surfactante é utilizada ou quando o surfactante está na presença de sal, o grau de dissociação de grupos polares torna-se menor e o sistema resultante pode ser do tipo a / o. Diluição com água pode aumentar a dissociação e a levar a um sistema o/a. Tensoativos iônicos são fortemente influenciadas pela temperatura. E, em altas temperaturas, são lipofílicos e formam sistemas a/o. A uma temperatura intermediária, microemulsão, por exemplo, convive com o excesso de água e de óleo e forma estrutura bicontinuas. (III) O comprimento da cadeia, o tipo e a natureza do cotensoativo. Os álcools de cadeia curta são amplamente utilizados como cotensoativos, e efetivamente promovem uma curvatura positiva, favorecendo a formação das microemulsoes, devido ao fato de reduzirem a tensão interfacial e aumentar a fluidez da interface. Aumentam também a

entropia do sistema atual influenciando na solubilidade das fases aquosa e oleosa [7, 28, 29].

PRODUÇAO E ENERGIA DE FORMAÇAO

Como explicado anteriormente, as microemulsões são termodinamicamente estáveis, e por isso podem ser simplesmente preparadas à partir de uma mistura de óleo, água, tensoativo e cotensoativo com agitação moderada ou leve aquecimento. No tocante à energia de formação e métodos de produção, em proporções adequadas dos componentes e, em condições de temperatura, pressão e força iônica constantes, o sistema microemulsionado forma-se espontaneamente, quando a energia remanescente da interface está próxima de zero ($\Delta G \rightarrow 0$)[12].

A relação entre o comportamento de fases de uma mistura e sua composição pode ser capturado com a ajuda de um diagrama de fases. A construção de diagramas de fase é demorada, principalmente quando o objetivo é exatamente traçar uma fronteira das fases. Técnicas como aquecimento e sonicação são frequentemente utilizadas para acelerar o processo, particularmente com os sistemas contendo tensoativos não iônico. As transições entre as diferentes fases traçadas nestes diagramas podem ser influenciadas pela adição de mais componentes, tais como fármacos ou eletrólitos, ou mudando a temperatura. Mudanças de fases das microemulsoes podem ocorrer através de uma série de diferentes estados estruturais, incluindo fases bicontinuas, sistemas lamelares e também multifásicos [22].

Ao contrário das microemulsões, as nanoemulsões são sistemas metaestáveis e sua estabilidade depende do método de preparação, são, portanto, requeridos métodos que cedam energia ao sistema. Esses métodos são divididos em: (I) Métodos de alta energia e (II) métodos de baixa energia.

Os métodos de alta energia envolvem o uso de equipamentos específicos, e a abordagem mais comum para emulsificação de alta energia é utilizar homogeneizadores de

alta capacidade de cisalhamento, de alta pressão ou geradores de ultra-som, também conhecidos como sonicadores. Enquanto os métodos de baixa energia utilizam as propriedades físico-químicas intrínsecas dos componentes da formulação para gerar as gotículas de emulsão na faixa nanométrica. Para fazer uso dessas abordagens de baixa energia, é necessário estudar a relação entre o comportamento das fases em equilíbrio do sistema inicial e das nanoemulsões resultantes [24, 26, 28, 30-32].

Devido às diversas vantagens dos métodos de baixa energia, em termos de rendimentos de formulação, e o potencial industrial na transposição de escala, tem havido um interesse real de pesquisa no desenvolvimento desses métodos e técnicas ao longo dos últimos anos. O primeiro método de baixa energia para formação de sistemas emulsionados em escala nanométrica foi a emulsificação espontânea, onde, a emulsão é formada como resultado de uma mistura de dois líquidos à temperatura ambiente. Um deles é uma fase aquosa pura, a outra é uma mistura de óleo, tensoativo e um solvente orgânico miscível com água. Os princípios e mecanismos que regem o processo de emulsificação é o deslocamento do próprio solvente do óleo para a fase aquosa que induz grande turbulência na interface água/óleo. Os dois líquidos, termodinamicamente estáveis por si só, são levados a um estado de não-equilíbrio quando são misturados. Assim, a rápida transferência de materiais hidrofílicos resulta em uma fase de grande aumento da área interfacial, dando origem ao estado metaestável da emulsão. Quando a difusão de solventes é rápida, a turbulência gerada produz gotículas em nanoescala. A fim de obter gotículas em escala nanométrica por este método, várias condições de trabalho são escritas na literatura e normalmente estão relacionados a uma relação de óleo e solvente, ao tipo de solvente orgânico utilizado, bem como o tipo de e proporção dos surfactantes [29, 32].

Outro método de baixa energia foi introduzido nos anos 60, é conhecido como método da temperatura de inversão de fase (método PIT). Óleo, água e surfactantes não-iônicos são todos misturados à temperatura ambiente. Em seguida, a mistura é aquecida gradualmente até a temperatura de inversão de fase do tensoativo. Como resultado, a

solubilidade dessas moléculas altera-se progressivamente da fase aquosa para a fase oleosa. Acima da temperatura de inversão de fases os surfactantes são totalmente solubilizado no óleo e, portanto, a mistura sofre uma inversão de fase, de um sistema óleo em água (o / a) para um sistema de uma água em óleo (a / o). Na temperatura de inversão de fases, a afinidade dos surfactantes para cada fase é semelhante, a curvatura interfacial é muito baixa, e conseqüentemente emulsões em escala nanométrica são formadas[24, 30].

Pode-se perceber que a formação de nanoemulsões é geralmente atribuída a instabilidades durante a fase de emulsificação. E, acredita-se que a presença de líquido cristalinos e / ou microemulsões bicontinuas desempenham papéis críticos. No entanto, uma vez que esses os sistemas podem passar por diversas fases durante a formação da nanoemulsão, é difícil precisar qual fase é fundamental[6].

Todos os sistemas emulsionados correspondem basicamente à uma dispersão coloidal de líquidos imiscíveis, e apesar de existirem atualmente diferentes tipos desses sistemas, o processo de emulsificação se baseia sempre no aumento de área interfacial, o que implica normalmente no aumento da energia livre de superfície[33, 34]. Este fenômeno, em condições de temperatura constante, pode ser descrito pela Equação:

$$\Delta G = G2 - G1 = \gamma i \times \Delta S \qquad \text{(Eq.1)}$$

onde, γi representa a tensão interfacial entre as fases aquosa e oleosa; S representa a entropia do sistema e G é a energia livre do sistema.

Diversas teorias são formuladas para explicar os mecanismos físico-químicos envolvidos na formação de um sistema emulsionado. Entretanto, não existe uma teoria universal de emulsificação, pois vários tipos de agentes de superfície podem ser incorporados à produção (tensoativos, polímeros e partículas), fazendo com que diferentes princípios direcionem a ação destas moléculas para alcançar a estabilidade do produto. As teorias existentes ainda não conseguem ser totalmente aplicadas aos sistemas reais e

práticos, apesar do conhecimento das microestruturas e dos vários mecanismos de estabilização terem aumentando significativamente[35].

Como mencionado, a emulsificação consiste em dividir uma das fases de um sistema heterogêneo em pequenas gotículas, no que resulta um aumento por vezes extraordinário da respectiva superfície, mas esse processo é contrariado pela tensão interfacial que os líquidos possuem. Esta propriedade representa a tendência que um líquido tem para reduzir sua área de superfície a um mínimo de energia potencial. Assim, para aumentar a superfície de um líquido qualquer, certa energia sob forma de trabalho (τ) deve ser desprendida para vencer a atração que a massa do mesmo líquido exerce sobre suas moléculas situadas à periferia. O fator mecânico, utilizado na fabricação de emulsões, representa este trabalho, mas o mesmo não é suficiente para permitir a obtenção de sistemas estáveis[34, 36].

A tensão superficial (σ) é definida como a força existente na superfície de um líquido, devido à atração entre as moléculas que se opõe à ruptura da superfície, e a tensão interfacial é a força necessária para romper a superfície entre dois líquidos imiscíveis. Essa é a força de tração que age sobre um elemento de superfície situado no plano tangente à superfície e que se opõe à dilatação desta. Essa grandeza é expressa em $dyn.cm^{-1}$. Fisicamente, a tensão superficial exprime a força por unidade de superfície com a qual as moléculas são atraídas ao interior do líquido. A maioria dos métodos para medir essa grandeza, são baseados em formas variadas da equação de Laplace, que descreve as diferenças de pressão que existem entre as interfaces curvas[37]. Esta diferença de pressão é dada, em Pascal (Pa), e pode ser resumida por:

$$\Delta p = \sigma(1/R_1 + 1/R_2) \qquad \text{(Eq.2)}$$

Onde:

σ (N.m-1) = Tensao surpeficial ou interfacial

R_1 e R_2 (m) = Raios da curvatura da superfície no ponto em questão

Para uma interface esférica onde $R_1 = R_2 = R$, tem-se:

$$\Delta p = 2\sigma / R \qquad \text{(Eq.3)}$$

Quando um líquido divide-se no interior do outro para a formação de um sistema emulsionado, forma-se uma fase interna, também denominada dispersa ou descontínua, rodeada por uma fase externa, dispersante ou contínua e isso implica no aumento da área interfacial, fazendo-se necessário o aumento da energia livre para a estabilização dessa mistura. Considerando que para emulsões farmacêuticas uma característica mínima de estabilidade é imprescindível, uma das alternativas utilizadas é o fornecimento de energia mecânica, de modo a manter a área interfacial aumentada. Além disso, outro tipo de estratégia de estabilização dessas dispersões é a utilização de agentes de superfície como surfactantes e /ou polímeros, que possuem a propriedade de diminuir a tensão interfacial entre as fases. Entretanto, esses compostos, não conseguem diminuir a tensão interfacial a ponto de contrariar totalmente a energia livre de superfície provocada pelo aumento da área interfacial. Por isso, as emulsões comuns são consideradas sistemas termodinamicamente instáveis e, durante o desenvolvimento tecnológico, procura-se utilizar meios que possam retardar pelo maior tempo possível a separação das fases[33, 34, 37].

A redução da tensão interfacial é alcançada com o emprego de moléculas de propriedades emulsionantes. De acordo com a essa teoria, uma emulsão pode ser então ser formada por dois líquidos imiscíveis se um agente que diminui consideravelmente a tensão interfacial for adicionado ao sistema. Os tensoativos, devido às suas características moleculares, se posicionam entre as interfaces do óleo e da água. Estes agentes também protegem o sistema contra a coalescência das gotículas, uma vez que são adsorvidos na interface. O processo de emulsificação clássico inicia-se quando um tensoativo adequado é dissolvido naquela que será a fase externa da emulsão final. Posteriormente, a fase interna é adicionada a esta, e promove-se a dispersão sob homogeneização[35].

A porção hidrofílica dos tensoativos orienta-se em direção à fase hidrofílica e a porção hidrofóbica em direção à fase lipofílica. A diferença relativa ao tamanho e força dos

grupos polares e apolares determina a capacidade de emulsificação do tensoativo. Geralmente, se as propriedades hidrofílicas dessas moléculas são levemente mais predominantes do que as propriedades hidrofóbicas, esta orientar-se-á na interface lipófilo-hidrófilo para que a porção hidrofóbica seja forçada para o centro e, a emulsão resultante será lipófilo-hidrófilo. Se o caráter hidrofóbico do tensoativo é mais predominante que o caráter hidrofílico, emulsões do tipo hidrófilo-lipófilo serão obtidas[27].

No caso das microemulsoes uma racionalização simplificada é apresentada abaixo. A energia livre de formação de microemulsão pode ser considerada depedente da capacidade do surfactante em reduzir a tensão da interface óleo-água e da mudança de entropia do sistema de tal forma que,

$$\Delta Gf = \Delta S\ \gamma\text{-}T/\ \Delta A \qquad \text{(Eq)}$$

Onde :

ΔGf onde é a energia livre de formação,

γ é a tensão da interface óleo-água,

ΔA é a variação na área interfacial,

ΔS é a variação de entropia do sistema que é efetivamente a entropia de dispersão,

E, T é a temperatura.

Pode-se perceber que quando uma microemulsão é formada a mudança de ΔA é muito grande devido ao grande número de pequenas gotículas formadas. Para uma microemulsão ser formada um valor negativo de γ é necessário, no entanto, é reconhecido que, embora o valor de γ seja positivo em todos os momentos, este valor é muito pequeno (da ordem de frações de Mn / m), e é compensado pelo componente entrópico. Existem também contribuições entrópicas decorrentes de outros processos dinâmicos como a difusão de surfactante na camada interfacial e na troca de surfactante monômero-micela. Assim, uma energia livre de formação negativa é alcançada quando grandes reduções da tensão na superfície são acompanhadas por mudanças entrópicas significativamente

favoráveis. Nesses casos, entende-se que microemulsificaçao é espontânea e a dispersão resultante é termodinamicamente estável[12, 22].

A utilização de microemulsões e nanoemulsoes está intimamente relacionada a sistemas para veicular a liberação de medicamentos, e é revisado constantemente em vários artigos cientificos, com especial destaque, esses sistemas são propostos como perspectivas futuras para várias moléculas insolúveis, anfifílicas ou que figuram um desafio para o desenvolvimento farmacêutico. As vantagens associadas a esses sistemas incluem a sua estabilidade termodinâmica para o caso das microemulsoes e cinética, no caso das nanoemulsoes; além disso, a claridade óptica e facilidade de preparação em ambos os casos também são de considerável importância.

A formulação de microemulsões para uso como sistemas autoemulsionates (SEDDS ou SMEDDS), ou como etapa inicial na produção de nanoparticulas, nanocapsulas e até nanoemulsoes tem sido bastante investigada [9, 23, 38, 39]. Vários trabalhos comprovam também a possibilidade de formular essas preparações para a maioria das vias de administração. Como foi discutido anteriormente, a presença de água ou outro co-solvente polar em uma formulação SEDDS pode significar que o concentrado é em si uma microemulsão. Ao destacar os fatores que predispõem microemulsões alguns autores observam que o processo de auto-emulsificação exige menos energia. A existência de microdomínios de polaridade diferente dentro da solução monofásica permite ser solubilizado e, ao mesmo tempo, tanto substancias solúvel em água quanto em óleo, se assim for desejado. Além disso, também é possível incorporar drogas anfifílicas nas microemulsoes, às vezes até levando a um aumento na extensão da existência da região de microemulsão[16-18].

Contudo, uma quantidade considerável de trabalhos fundamentais que caracterizem o comportamento físico-químico de microemulsões precisa ser realizada antes que

possamos desfrutar por completo do potencial desses sistemas como carreadores polivalentes de fármaco.

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Deuxième partie

TRAVAUX EXPERIMENTAUX AU BRESIL

A NEW INSIGHT ABOUT PHARMACEUTICAL DOSAGE FORMS FOR BENZATHINE PENICILLIN G.

A NEW INSIGHT ABOUT PHARMACEUTICAL DOSAGE FORMS FOR BENZATHINE PENICILLIN G. Journal of Basic and applied Pharmaceutical Sciences. v.27, p.21 - 26, 2006.

SILVA, K. G. H., XAVIER JÚNIOR, F.H., FARIAS, I. E. G., CALDAS NETO, J. A., Silva, A. K. A., NAGASHIMA JUNIOR, T., ARAÚJO, I. B., OLIVEIRA, A. G., MEDEIROS, A. C., EGITO, E.S. T.

La pénicilline G est une pénicilline naturelle. Bien qu'elle soit découverte en 1928 et utilisé en clinique depuis les années 40, cette molécule occupe toujours une place importante dans le traitement des infections causées par les streptocoques. Cette molécule tient une place importante dans l'histoire de la pharmacologie, car elle a déjàété le premier médicament réellement efficace contre de nombreuses maladies graves telles que la syphilis et les infections à staphylocoques. Elle est encore largement utilisée aujourd'hui, même si des nombreux types de bactéries sont devenus résistants.

Pourtant, les propriétés physico-chimiques de cette molécule ne sont pas très adaptées à l'élaboration des systèmes pharmaceutiques appropriés pour le traitement à long terme et ceci est suivant la raison de l'échec du traitement. De nouvelles technologies peut être utilisées pour developper des nouvelles formes pharmaceutiques plus performantes.

Au cours de la mise au point de systèmes pharmaceutiques nouvelles, l'étape de pré-formulation est toujours important. Ces études sont basées principalement sur le développement des formulations avec une stabilité physique et chimique suffisante, dont le profil biopharmaceutique est adaptée au but. Les analyses du coefficient de partage et de la solubilité dans un milieu liquide sont parmi des tests les plus importants dans cette phase.

L'objectif principal de cette thèse est de développer un système de liberation contrôlée pour la pénicilline G benzathine. Toutefois, un test préliminaire était nécessaire avant de commencer le développement réel de ce système. Dans cet essai, un systeme micellaire de PenGB et désoxycholate de sodium a été élaboré et analysé. Les résultats démontrent la capacité de ce système pour augmenter la solubilité de PenGB.

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A new insight about pharmaceutical dosage forms for benzathine penicillin G.

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ABSTRACT

In this work, a micellar system of benzathine penicillin G (BPG) in sodium deoxycholate (NaDC) was developed and evaluated physicochemically. The solubility profile of the drug in water and buffer solutions at various pH was determined, as well as its n-octanol/water partition coefficient. The Critical Micellar Concentration of NaDC and its ability to incorporate BPG were also assessed. The study was carried out at low and high ionic strength which was adjusted by the addition of sodium chloride. The results demonstrated the ability of the micellar system to incorporate BPG, as well as to increase its apparent solubility in water. The enhancement of the solubility of BPG by the presence of NaDC micelles could be analyzed quantitatively within the framework of the pseudo-phase model. Concentration analysis showed that the micellar system could attain up to 90% incorporation of BPG. The incorporated drug is expected to exhibit improved stability, since the antibiotic enclosed in the hydrophobic core of micelles is rather shielded from the aqueous external environment.

Keywords: Benzathine Penicillin G; micellar solubilization; micelles; pre-formulation; sodium deoxycholate.

INTRODUCTION

Benzathine penicillin G (BPG) is natural penicillin whose molecular structure is $C_{48}H_{58}N_6O_8S.4H_2O$. BPG is chemically designated as N, N-dibenzyl ethylenediamine dipenicillin and its molecular mass is 981.19 daltons. It occurs as a white, crystalline, odorless powder, and is very slightly soluble in water and sparingly soluble in alcohol. BPG concentrations are described in international units (IU), in which 1mg corresponds to 1,211 IU of penicillin (Shulman & Gerber, 2004). The antibacterial activity of BPG is mainly against Gram-positive bacteria (including actinomycetes) and some Gram-negative Cocci, as well as some spirochetes (Parfitt, 1999). BPG is widely used in the treatment of numerous infectious diseases, especially those related to obstetric and gynecologic conditions. In general, penicillin is effective in the treatment of localized skin and soft-tissue infections of the nose, throat, lower respiratory tract and genitourinary tract. It is also commonly used in prevention of bacterial endocarditis prevention, prophylaxis during gastrointestinal and/or genitourinary procedures, and treatment of rheumatic fever (Miller, 2002).

According to the American Academy of Pediatrics, the American Heart Association, the Infectious Diseases Society of America and the World Health Organization the first-line treatment for streptococcal infections consists of monthly injections of 1,200,000 UI of BPG (Currie, 1996; Kassem, et al., 1996). However, lack of patient compliance has meant in treatment failure (Currie, 1996; Carapetis et al., 2000; Peloso et al., 2003).

Sustained-release dosage forms could be a promising means of overcoming this drawback in relation to rheumatic fever treatment. When they are used, several come into play (Peloso et al., 2003), for example: less frequent dosing, improved bioavailability, reduced concentration, increased absorption and minimization of adverse drug reactions.

Pre-formulation studies are a preliminary stage in the development of new drug dosage forms. Such studies mainly focus on developing physically and chemically stable formulations, whose biopharmaceutical profile is suitable for their purpose. Partition coefficient analysis and solubility tests in liquid media are some of the most important tests at this stage (Blasco et al., 2001; Granero et al., 1999).

The drug dosage form for BPG which is currently available on the pharmaceutical market is an intramuscular injection suspension. Due to its variable physical properties, such as solubility degree, viscosity and crystal size, deviations on its bioavailability and release profile have been reported (Currie, 1996; Carapetis et al., 2000).

Micelles and other related systems have had considerable success in changing some of the physical and chemical properties of drugs, e.g. solubility and stability (Oliveira et al., 1990; Oliveira et al., 1991; Oliveira & Chaimovich, 1993). Micellar and directly-related systems are dynamic colloidal supramolecular aggregates containing surfactant molecules that can efficiently dissolve water-insoluble drug compounds (Oliveira et al., 1997; Oliveira & Scarpa, 1999). The micelization process takes place when the surfactant concentration exceeds the Critical Micellar Concentration CMC (Tascioglu, 1996). This is the results

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from an induced interaction of intermolecular forces, including electrostatic and steric repulsions, hydrogen bonds, and Van der Waals interactions. This approach is of particular interest in the pharmaceutical field, given the ability of these systems to increase the solubility of hydrophobic drugs (Rangel-Yagui et al., 2005). Besides, this technique involves easily-controlled parameters, as the factors that change the properties of micelles are well established. In this study the physicochemical properties of BPG were evaluated. The CMC was determined for sodium deoxycholate (NaDC) in aqueous solution, as well as its effect of sodium chloride on its CMC value. It was aimed to develop a micellar system containing BPG, using data obtained on ideal surfactant concentration need to dissolve the drug.

MATERIAL AND METHODS

Determining solubility profile of drug in liquid media is a critical stage in the development of new drug delivery systems. In fact, the profile plays a major role in drug dissolution, which is a rate-limiting step for drug absorption (Blasco et al., 2001). Another major point to consider is the analysis of the n-octanoll/water partition coefficient. This parameter is largely used to determine the degree of hydrophobicity in pharmacological models, as well as to study toxic properties (Roberts, 2002). In addition to that, it provides a theoretical approach to the partition of molecules in biological membranes during absorption processes.

In the study of micellar systems, the determination of both the CMC of the surfactant and the parameters that can modify its value are mandatory steps.

Drug Solubility Determination

In the experiments, a supersaturated solution of BPG was used, as described in the equilibrium method proposed by Granero et al. (1999).

Solubility in water and n-octanol

Excess of drug (250mg) was added to 5mL of solvent (distilled water or n-octanol), and was stirred magnetically for 24 hours at 25 ^{o}C. Afterwards, the mixture was allowed to rest for 12 hours. As equilibrium was reached, the samples were filtered through by using a 0.8μm membrane and the drug concentration in the filtrate was analyzed by UV-Vis spectrophotometry.

Solubility in different pH

The pH values of the buffer solutions varied from 5.0 to 9.0 to obtain solubilities in acid, neutral and alkaline conditions. Samples were stirred in an ultrasound bath for 3 hours to obtain saturated solution and allowed to rest for 1.5 hour. After that, the concentration of the dissolved BPG was determined by UV-Vis spectrophotometry.

Partition coefficient determination (log P)

This parameter was determined in n-octanol/water mixture (Roberts, 2002; Sangster, 1997). The two phases of solvents were stirred until equilibrium was reached, then an accurately weighed amount of BPG dissolved in a minimal amount of methanol was added. The mixture was stirred for 1 hour, and allowed to rest for 36 hours for phase separation. An aliquot of each phase was withdrawn by pipette, and analyzed by ultraviolet spectrophotometry. The partition coefficient was taken as the ratio of the drug concentration (w/v) in the n-octanol and water phases, respectively.

Determination of NaDC Critical Micellar Concentration

The CMC of sodium deoxyxcholate (NaDC) was evaluated by determining the electric conductivity of solutions, in which the concentration of the surfactant varied from 0.8 to 18.1mM. The results were plotted as a function of NaDC concentration and the CMC was taken as the point of intersection of the curves.

To evaluate the effect of ionic strength on the CMC, the electric conductivity of NaDC solutions was determined in saline solutions using the same method as for the solutions in water.

Preparation of micellar solutions of BPG

In order to prepare BPG micellar solutions a solution of NaDC containing NaCl was used. The drug incorporation took place under stirring in an ultrasound bath. Macroscopic analysis was performed against black background. Drug concentration was assessed by UV-Vis spectrophotometry after calibration of the apparatus. The concentration of BPG in micellar solution was established by subtracting its concentration in the supernatant liquid from the bulk (nominal) value.

RESULTS

The results for the solubility of the BPG in some n-octanol, water and buffers at several pH values are displayed in table 1, together with the n-octanol/water partition coefficient.

Table 1. Physicochemical properties of BPG and NaDC micelles.

BPG Solubility [mM]					log P
Water	Octanol	pH 5.4	pH 7.4	pH 8.9	
0.16	0.026	0.057	0.485	1.13	1.12

Note that the solubility of benzathine penicillin G in water was very low, decreasing to a value 6.2 times lower in n-octanol (Table 1). In addition, the solubility depended on pH increasing 8.5 fold as the pH enhanced from 5.4 to 7.4, and a further 2.3 fold from pH 7.4 to 8.9. The n-octanol/ water partition coefficient was also low.

The results of the CMC determination for sodium deoxycholate are described in Table 2 and show clearly that at high ionic strength due to sodium chloride concentration of the saline solution, the value of the CMC was much lower than in water favoring micelle formation at lower surfactant concentrations.

Table 2. Effect of NaCl on the Critical Micellar Concentration of NaDC. [NaCl] = 0.09g/mL (163.63mM)

CMC of NaDC [mM]	
Aqueous solution	**Saline solution**
3.6	0.8

The CMC was easily determined from the conductivity measurements at various NaDC concentrations, since that at CMC the curve has a marked discontinuity and a significant change in the inclination between the straight lines (Figures 1 and 2).

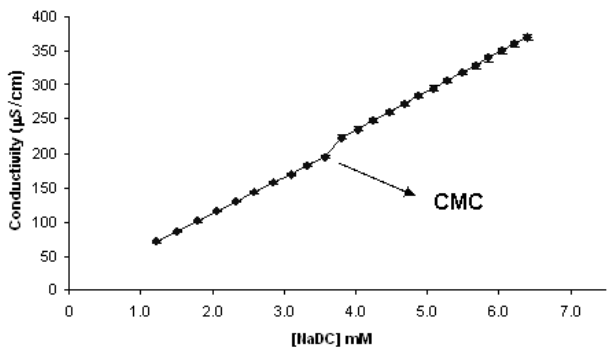

Figure 1. Determination of the Critical Micellar Concentration of NaDC in aqueous solution. (25°C)

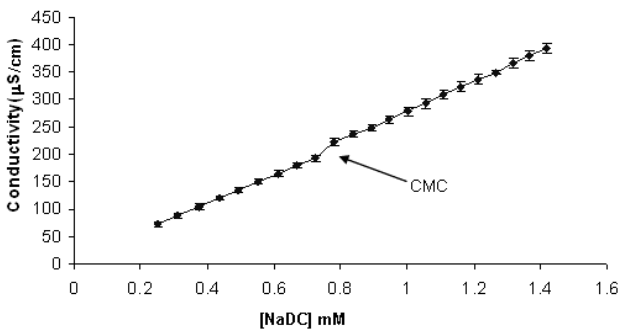

Figure 2. Effect of 0.9% (w/v) sodium chloride on the Critical Micellar Concentration of NaDC. (25°C).

Considering that in the presence of saline solution the CMC of the NaDC was about 0.8 mM, the experimental determination of micellar incorporation of BPG was conducted at NADC concentrations below and above CMC (Table 3).

Table 3. Effect of the NaDC concentration on the solubilization of BPG into micellar solutions in saline.

Sample	NaDC (mM)	BPG		Abs	Incorporation % w/v
		Bulk	Supernatant		
1	0.49	5.81	2.83	0.14	48.76
2	0.98	6.01	3.51	0.15	58.33
3	1.96	6.15	4.03	0.20	65.60
4	2.45	5.98	5.00	0.25	83.67
5	2.94	5.84	5.28	0.26	90.28
6	3.92	6.32	5.63	0.28	89.10

The concentration of dissolved BPG increased with increasing surfactant concentration up to a plateau (Figure 3). This incorporation profile is typical of micelle-modified drug solubility where the drug solubility in the micellar pseudo-phase is higher than that in the aqueous phase.

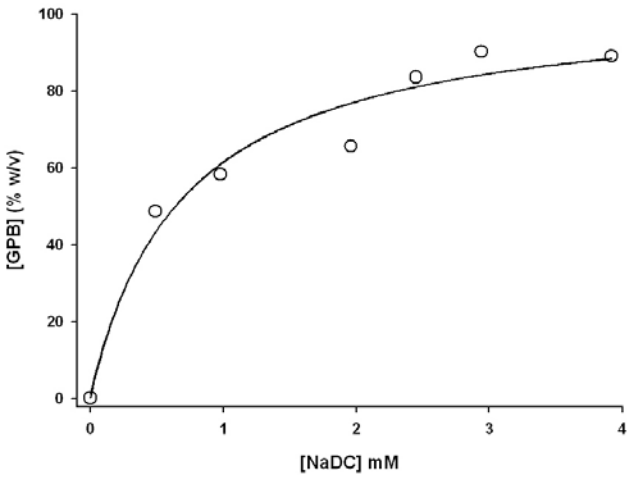

Figure 3. Effect of NaDC on the micellar incorporation of BPG in aqueous solution containing sodium chloride 0.9 % w/v (163.63mM). See table 3 for details. Solid line calculated from equation 1.

This increase in the solubility suggests that BPG may be distributed between the aqueous and micellar pseudo-phases. Then, the data in the Figure 3 can be analyzed quantitatively by the expression of the pseudo-phase model (Oliveira et al., 1991).

$$BPG_d = \frac{BPG_w + BPG_m \cdot Ks \cdot [NaDSC]}{(1 + Ks) \cdot [NaDSC]} \qquad (1)$$

Where the subscript d, w, and m refer to the concentrations of total dissolved BPG, BPG dissolved in the water phase and BPG dissolved in the micellar phase, respectively. Ks is the constant that describes the incorporation of the BPG into micellar system. The value of BPG_w was obtained from the solubility of BPG in the absence of NaDC.

The calculated value of Ks was about 106, demonstrating clearly the incorporation of the BPG into the micellar system.

This feature can be seen in the photographs in Figure 4, which shows that at low surfactant concentrations, BPG is partially dissolved, generating opaque and semi-transparent dispersions which are completely dissolved at high surfactant concentrations.

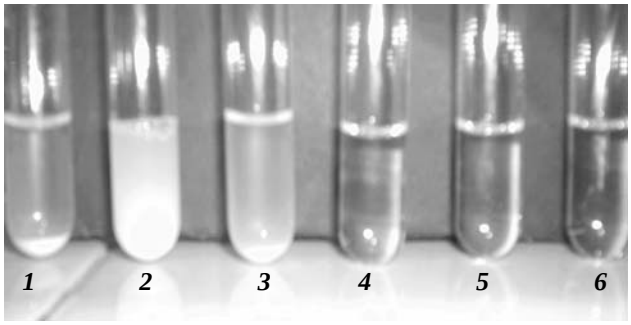

Figure 4. Macroscopic aspect of the system during BPG incorporation into micellar aqueous NaDC. (**1**) pre-micellar dispersion of BPG; (**2-6**) Stages in incorporation of BPG into NaDC micelles at various concentrations of NADC (see Table 1).

DISCUSSION

In the experiments of BPG solubility in different solvents a high degree of insolubility was observed in both lipophilic and hydrophilic media. In fact, these data agree with those in the literature (Kreuzig, 1982), and suggest the amphiphilic character of the drug molecule. However, it was observed that the solubility increased with the pH (Table 1), possibly due to the fact that BPG is an acid drug, with the pKa of the carboxyl group around 2.65, depending on the solvent. Thus, in the range of pH studied the equilibrium is displaced towards ionized species, which are fast hydrated, becoming more soluble (Atwood & Florence, 1985).

The determination of partition coefficient provided log P for the n-octanol/water system. The low value of log P indicates low partition capacity and medium-low hydrophobicity. Thus, the value described in Table 1 reveals a medium ability of the drug to diffuse to the hydrophobic core of the micelles.

Surfactant molecules, constituted by a polar head-group and a hydrocarbon-chain longer than eight methylene groups, associate spontaneously in water to form dynamic aggregates denominated micelles (Oliveira & Chaimovich, 1993). In dilute solutions below the CMC these molecules are dispersed individually in the medium as monomers and such solutions exhibit ideal physical and chemical properties. As the surfactant concentration increases the properties deviate from ideal indicating the aggregation of monomers above the CMC forming micelles, provoking the differentiated properties of the micellar pseudo-phase (Tascioglu, 1996).

When the conductivity is plotted against surfactant concentration, the data points fit on two different lines (Figures 1 and 2). The first corresponds to concentrations below the CMC, in which only surfactant monomers exist in the solution (Atwood & Florence, 1985; Oliveira & Chaimovich, 1993). At higher concentrations, micelles appear, and conductivity curve suffers discontinuity at the CMC. The second parte of the curve has a different slop. The determination of the CMC was a first step in the BPG incorporation study, used to indicate exactly the surfactant concentration at which the micelles were formed in solution.

Addition of inorganic salts is able to change micelle size and shape. Therefore, ionic strength influences the solubilization rate of drugs. In fact, salt tends to reduce the mutual electrostatic repulsion of the head groups of surfactants, which become more hydrophobic. This effect induces surfactant aggregation at lower concentrations. As a result the CMC is lower and the final cost of these systems is reduced (Tascioglu, 1996). This phenomenon was observed here for NaDC (Figure 2), and also found to function in BPG micellar system. The incorporation of BPG into the micelles was feasible by a simple and fast method that was able to increase drug incorporation markedly in solutions above the CMC (Table 3 and Figure 3).

The value of Ks calculated from the plot of the data by equation 1 (Ks=106) demonstrated that the incorporation of BPG into the micellar system is due mainly to the hydrophobic property of the drug molecule. It is known that in several cases the effect of micelles on drug solubility can be attributed to the free energy of activation resulting from transfer of the substrate to a medium of lower dielectric constant (Oliveira et al., 1991). Indeed, our results show clearly that the solubility of BPG was increased when the antibiotic was transferred to the low dielectric constant medium of the hydrophobic core of the NaDC micelles. In addition, this value for Ks is in agreement with the partition coefficient (log P = 1.12) indicating that antibiotic molecule is moderately lipophilic.

The determination of the analytical concentration of the drug while [NaDC} was raised showed that the percentage of incorporated BPG could reach about 90% (Table 3). Hence, micellar solutions appear to be a promising approach improving the physical-chemical properties of BPG for therapeutic use. The dibenzyl ethylenediamine moiety, added to penicillin G to produce BPG, delays its release by the formation of a water-insoluble deposit at the site of injection, due to molecular aggregation (Martinez-Ladeira, et al., 2004).

The incorporation of BPG into micellar systems is intended to reduce the incompatibility of this molecule with water (Blaha et al., 1976). Its resistance against the hydrolytic and enzymatic degradation may be also be improved. As a consequence, its pharmacological effect may be achieved at lower doses. Besides, assuming that drug molecules do not undergo degradation, they will remain pharmacologically active for longer. The incorporation of BPG in micellar solutions was feasible by a simple and fast method that was also able to increase markedly the amount of incorporated drug in solutions above the CMC. Hence, micelle solutions seem to be a promising approach to improving the physical-chemical properties of BPG for therapeutic use.

RESUMO

Novas perspectivas para sistemas de liberação para a penicilina G benzatina

O objetivo desse trabalho foi o de desenvolver e avaliar do ponto de vista físico-químico um sistema micelar de penicilina G benzatina (BPG) em desoxicolato de sódio (NaDC). Foram estudadas as características físico-químicas da BPG quanto à solubilidade em água e em soluções tampões com diferentes pHs, além do coeficiente de partição octanol-água. Foram avaliadas as propriedades da concentração micelar crítica (CMC) das soluções micelares de Desoxicolato de sódio (NaDC) em baixa e alta força iônica provocada pela presença de cloreto de sódio. O estudo da incorporação da BPG em soluções micelares de NaDC usando várias concentrações de NaDC também foi realizado. O aumento da solubilidade da BPG provocada pela presença de micelas de NaDC foi analisada quantitativamente pelo formalismo do modelo de pseudo-fase. Houve indicação da aplicabilidade do sistema micelar estudado quanto à incorporação de penicilina e aumento se sua solubilidade aparente, com taxa de incorporação de até 90%. Espera-se que a formulação micellar de BPG apresente melhor estabilidade, considerando-se que o antibiótico incorporado na região hidrofóbica das micelas está protegido do meio aquoso externo.

Palavras-chave: penicilina G benzatina; solubilização micelar; micelas; pré-formulação; desoxicolato de sódio.

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STATIONARY CUVETTE: A NEW APPROACH TO OBTAINING ANALYTICAL CURVES BY UV-VIS SPECTROPHOTOMETRY

STATIONARY CUVETTE: A NEW APPROACH TO OBTAINING ANALYTICAL CURVES BY UV-VIS SPECTROPHOTOMETRY. Phytochemical Analysis. 2009 Jul;20 (4):265-71.

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Une méthode d'analyse comprend la gestion de l'analyse et toutes les étapes nécessaires pour effectuer chaque test analytique. Pour une analyse chimique quantitative la séquence d'étapes inclut la méthode de dépistage, l'obtention de l'échantillon, le traitement des échantillons, l'élimination des interférences, d'étalonnage et de mesure de la concentration, le calcul des résultats et évaluation des résultats par l'estimation de la fiabilité. Des techniques analitiques actuellement disponibles pour l'analyse des médicaments dans des formulations pharmaceutiques sont constamment à l'examen et la validation. Toutefois, la méthode actuelle utilisée pour l'étalonnage des méthodes spectrophotométriques présente certains inconvénients. L'objectif de cette travail était de développer une nouvelle technique d'étalonnage: Cuve Satationaire permettant de résoudre les inconvénients de la méthode traditionnelle. Les méthodes ont été comparées et les sources possibles de variation entre eux ont été évalués.

Research Article

Phytochemical Analysis

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Stationary Cuvette: a New Approach to Obtaining Analytical Curves by UV–VIS Spectrophotometry

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ABSTRACT:
Background – Investigations in the field of pharmaceutical analysis and quality control of medicines require analytical procedures that achieve suitable performance. An analytical curve is one of the most important steps in the chemical analysis presenting a direct relationship to features such as linearity.
Objective – This study has the aim of developing a new methodology, the stationary cuvette, to derive analytical curves by spectroscopy for drug analysis.
Methodology – The method consists basically of the use of a cuvette with a path length of 10 cm, containing a constant volume of solvent in which increasing amounts of a stock solution of the sample are added, droplet by droplet. After each addition, the cuvette is stirred and the absorbance is measured. This procedure was compared with the currently used methodology, which requires a labour-intensive dilution process, and possible sources of variation between them were evaluated.
Results – The results demonstrated that the proposed technique presented high sensitivity and similar reproducibility compared with the conventional methodology. In addition, a number of advantages were observed, such as user-friendliness, cost-effectiveness, accuracy, precision and robustness.
Conclusion – The stationary cuvette approach may be considered to be an appropriate alternative to derive analytical curves for analysing drug content in raw materials and medicines through UV–VIS spectrophotometry.

Keywords: Analytical curve; UV–VIS spectrophotometry; stationary cuvette; validation method; pharmaceutical analysis

Introduction

The detection of a substance at low concentrations within a matrix of another (e.g. a dilute solute in solution) is a typical task performed in analytical science. In many cases, the manipulation of a sample (e.g. by concentration adjustment) is imperative, so the key issue relates to the sensitivity and selectivity of the current measurement techniques (Nigmatullin *et al*., 2005). To detect a compound from a pharmaceutical or a phytochemical product or other complex matrix from the vegetal and biological fields, the extraction process is a crucial step. Several methods could be used for this extraction process. Among them, surfactant association (Shokrollahi *et al*., 2008), preliminary saponification (Bunea *et al*., 2008), and solvent and specific extraction techniques (Hubert *et al*., 1999; Grubesic *et al*., 2005) have been the most widely used.

Investigations in the field of pharmaceutical and phytochemical analysis, and quality control of medicines, require analytical procedures with acceptable performance characteristics. The quality of analytical measurements is of the utmost importance. The concern for assuring the reliability and quality of analytical results has encouraged the publication of guidelines to validate analytical procedures (ICH, 1996; USP, 2004).

The validation of analytical methods is the process by which it is assured that the characteristics of a method match those required for analytical purposes. This process is somewhat expensive, time-consuming and labour-intensive. Therefore, some authors have proposed a stage of pre-validation as a first step towards the validation of analytical methods. The main step in the pre-validation study is to obtain a suitable calibration model by means of linearity and robustness analyses, which evaluate the stability and reproducibility of the method (Hubert *et al*., 1999, Soares *et al*., 2004, 2007; Grubesic *et al*., 2005).

Actually, calibration is the crucial step related to the validation and pre-validation processes, and is directly associated with parameters such as linearity (Garcia *et al*., 2003). Analytical calibration intends to correlate the value of an analytical signal obtained from sample analysis into data concerning the concentration of an analyte present.

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The most suitable model to fit analytical curves is usually simple linear regression analysis, which is based on statistic tools to investigate the relation between two or more parameters and is used to predict the results of future analyses. In such an analysis, estimations to achieve optimal fitting of the model are carried out by means of the computation of regression coefficients, which in turn are derived from the method of least squares (ICH, 1996; USP, 2004).

Besides calibration, other key points must be considered. Validating an analytical method requires an investigation into whether it runs correctly when subjected to slight changes in standard conditions. Despite the fact that the International Union of Pure and Applied Chemistry (IUPAC) only defines the term 'robustness' (ICH, 1996), the *United States Pharmacopeia* (USP) also introduced 'ruggedness' (USP, 2004). These terms indicate to what extent the quality of data is independent of small changes in the procedure. Robustness/ruggedness assays indicate parameters that should be taken into account in the development step, and the ones that directly influence the method. These analyses must be carried out during the method development stage or in the beginning of the validation step (ICH, 1996; Grdinic and Vukovic, 2004; USP, 2004).

Phytochemical products also need a system with high performance to analyse them. In fact, recent interest in botanical extracts has led to an increased requirement for efficient methodologies for the assurance of standardisation, reproducibility, efficacy, safety and quality of those varieties of raw materials.

Kalanchoe brasiliensis (Comb.), Crassulaceae family, is a medicinal herb largely found in the southeast of Brazil. In folk medicine, this natural product is used extensively to treat wounds, abcesses, inflammation and gastrointestinal ulcers. Its leaves contain several classes of chemical compounds, such as bufadienolides, terpenoids and flavonoids, as well as polysaccharides and ascorbic acid (Mourão *et al*., 1999; Ferreira *et al*., 2000; Costa *et al*., 2006). Because of their wide distribution, flavonoids, a group of polyphenolic compounds broadly distributed as secondary metabolism in plants and pharmaceutical, cosmetics and food industries, are eligible as chemical markers for this species of plant (Soares *et al*., 2003).

This study is intended to develop a novel interpolative methodology using a stationary cuvette to obtain analytical curves for drug and phytochemical products analysis by UV–VIS spectrophotometry. The proposed methodology was compared with the currently used one with respect to validation parameters and ruggedness for quantifying penicillin G benzathine, hydrocortisone and ibuprofen as well as *K. brasiliensis* extract.

Experimental

Apparatus. Shimadzu (Paris, France) UV 1650 and Varian (Palo Alto, CA, USA) EL 96023131 spectrophotometers with 1 cm (NSG, Farmingdale, NY, USA) and 10-cm (Hellma, São Paulo, SP, Brazil) path length quartz cells were used for absorption measurements.

Chemicals. Methanol, ethanol (JT Baker, Phillipsburg, NJ, USA), penicillin G benzathine (PenGB) (Sigma-Aldrich, St. Louis, MO, USA), hydrocortisone and ibuprofen (Deg, São Paulo, SP, Brazil) were of analytical grade, and distilled water was used throughout.

Plant material. The leaves of *K. brasiliensis* were collected in Macaíba (Rio Grande do Norte, Brazil). The raw material was identified by Dr. Maria Iracema Bezerra Loiola and a voucher specimen was deposited at the herbarium of the Federal University of Rio Grande do Norte, Natal, Brazil, under the registration number 5468.

Validation of the stationary cuvette method. In order to validate the stationary cuvette method, PenGB was used as a model drug. The linearity, precision, accuracy and ruggedness were evaluated. Different analysts repeatedly performed the experimental set-up of validation on different days using two different instruments. For testing the usefulness of the method, two extra drugs presenting different physicochemical and pharmacological properties were chosen. Hydrocortisone and ibuprofen were then evaluated in comparison with PenGB, and linearity and ruggedness tests were carried out.

Preparation of drug solutions. Stock solutions (w/v) of drugs were prepared by dissolving them in suitable solvents (Table 1). A drug stock solution (1000 μg/mL) was obtained in a 10 mL volumetric flask. The diluted solutions were prepared by diluting the stock solution using the same solvent. For the traditional method, the dilution was carried out in volumetric flasks. For the stationary cuvette method, stock solutions were added to a stationary volume of solvent into a larger (100 mm) analytical quartz cuvette. The experimental set-up was repeatedly performed on different days, with different instruments, and by different analysts. Freshly prepared drug solutions were always used.

Preparation of extract of *K. brasiliensis*. The plant extract solution was prepared by the turbo-extraction method in the proportion of 1:1 (w/v) using a 50% (v/v) ethanol solution as solvent. The flavonoids from this extract were complexed with 5% (w/v) aluminium chloride solution for 30 min. A scan-wave

Table 1. Absorbance detection wavelength of PenG B, hydrocortisone, ibuprofen and *K. brasiliensis* extract using conventional (1 cm path length quartz cell) and stationary cuvette methods (10 cm path length quartz cell)

Drug	Solvent	λ (nm)		Concentration (mM)
		Conventional	Stationary cuvette	
PenGB	Methanol	258 (Kreuzig, 1982) 254 (Santos-Magalhaes *et al*., 2000)	258	1.00
Hydrocortisone	Ethanol	238 (Hajkova *et al*., 2003)	242	3.4×10^{-2}
Ibuprofen	Ethanol	273 (Farrar *et al*., 2002)	274	5.00
K. brasiliensis extract	Ethanol 50%	410	410	—

spectrum of this solution, from 200 to 500 nm, was obtained to evaluate the maximum of the extract solution, which was found to be 410 nm. This general procedure is widely recommended by several of the official Codex to quantify the total flavonoid contents in plant extracts, after complex formation between aluminium chloride and flavonoids (which contain hydroxyl groups at C3 and C5 or ortho-dihydroxyl groups) (Mabry *et al*., 1970).

Analytical curves of samples. Prior to sample analyses, UV–VIS absorption spectra were obtained to identify the best detection wavelength for each tested molecule in appropriate solvents and concentrations as shown in Table 1. Parameters such as temperature, concentration, and presence of interfering substances were controlled.

Analytical curves using traditional method. In the conventionally used methodology (Adams and Bergold, 2001; Patravale *et al*., 2001; Soares *et al*., 2004; Saif and Anwar, 2005; Dost and Tokul, 2006), stock solutions of accurately weighed PenG B (1000 μg/mL), ibuprofen (1000 μg/mL), hydrocortisone (1000 μg/mL), and a complexed solution of *K. brasiliensis* (10% v/v) were prepared with an appropriate solvent (Table 1) and several dilutions were carried out at concentrations of 0.2–0.8, 0.97–3.89 and 0.01–0.02 mM and, for plant extract **4**, at 14 g/mL, respectively. The samples were analysed by UV–VIS spectroscopy to determine the absorbance at the detection wavelength using 1 cm path length quartz cells.

Analytical curves using stationary cuvette method. With respect to the proposed methodology, the samples were prepared from a stock solution by increasing sample concentrations, i.e. a constant amount of stock solution was added to a stationary volume of solvent in a large analytical quartz cuvette. The analyses were carried out in a 10 cm path length quartz cell containing 20 mL of the solvent. The absorbance was assessed at each addition of a preset volume (100 μL and 20 μL) of the drug stock solution and the complexed plant extract solution, respectively.

The analytical curves were plotted as a function of the drug solution concentration and the measured absorbance. The absorbance of these solutions was measured at the specific detection wavelength of each drug or plant extract (Table 1). The coefficient of determination, the line regression and the regression coefficients were obtained by the standard linear regression method.

Precision and accuracy. The intermediate precision and accuracy of the method was analysed according to the current legislation for validation of analytical methods (USP, 2004). Both parameters were evaluated in terms of the relative standard deviation (RSD%), whose maximum acceptable value was set at 5%. The latter was determined by analysing the agreement of the estimated results with the nominal values. Inter-run assays were performed by three different analysts using three different samples. The relationship between the experimental mean concentration and the corresponding theoretical one was then established. A statistical difference between means was considered to be up to 15% (USP, 2004).

Ruggedness evaluation. In order to carry out the ruggedness evaluation, the analyses were performed by three different analysts in two different laboratories with different instruments. Distinct optical path length cuvettes (1 and 10 cm) were used.

Applicability. In order to evaluate the applicability of the stationary cuvette method, two supplementary drugs (hydrocortisone and ibuprofen) and an extract of *K. brasiliensis* were analysed by the proposed methodology. Linearity results were then compared with those obtained by the conventional method. Ruggedness tests related to analysts and days were also carried out.

Statistical analysis. Statistical analyses were conducted through linear regression, Student's *t*-test and ANOVA using the software 'Statistic' (version 5, StatSoft Inc., São Caetano do Sul, SP, Brazil) and 'Microsoft Excel 2000' (Microsoft Corporation, New York, NY, USA).

Results and Discussion

Absorption spectra of drugs

The detection wavelength is considered to be that at which maximum absorbance of a substance is observed (Skoog *et al*., 1997). Concerning PenGB, the characteristic peak of this molecule was considered to be a shoulder at 258 nm (Kreuzig, 1982). The data related to the UV absorption spectra of PenGB, ibuprofen and hydrocortisone, and *K. brasiliensis* extract are shown in Figs. 1 and 2.

In order to obtain a reproducible absorbance–concentration relationship, the wavelength of maximum absorbance was

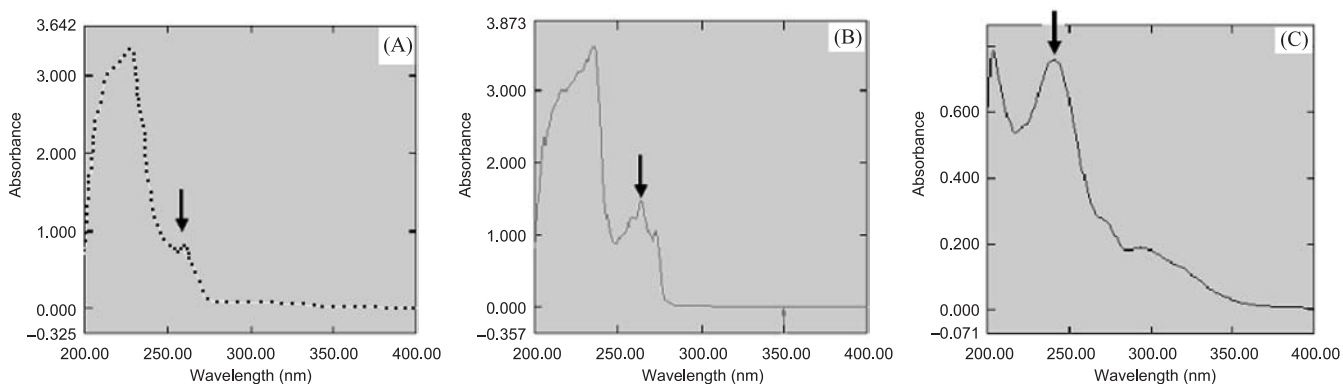

Figure 1. Absorption spectra of PenGB (A), ibuprofen (B) and hydrocortisone (C) at 1, 5 and 3.4×10^{-2} mM, respectively. (The arrow indicates the detection wavelength at which maximum absorbance was observed.)

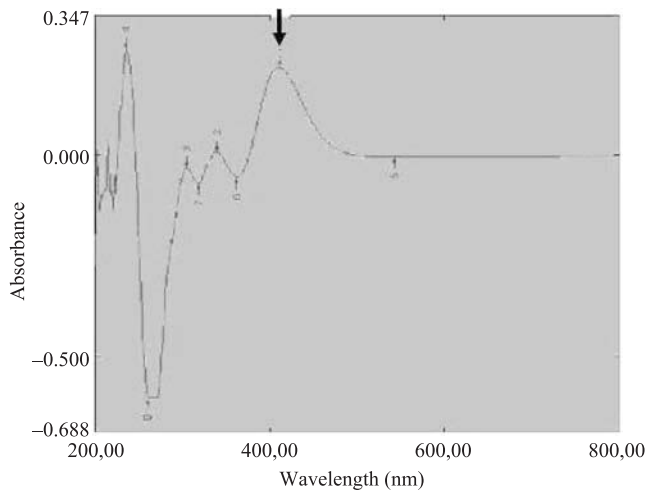

Figure 2. Absorption spectra of the diluted (5:1) plant extract solution which was complexed with aluminium chloride at 5% (v/v). (The arrow indicates the detection wavelength at which maximum absorbance was observed.)

considered. As the absorbance variation per concentration unit is more noticeable at this point, maximum sensitivity is assured. Furthermore, the absorption curve is rather smooth at maximum absorbance. As a consequence, a good agreement with Beer's law and an improved reliability are achieved. Additionally, imprecise wavelength adjustment by the equipment is kept to a minimum (Skoog *et al*., 1997).

Concerning the observed detection wavelengths of the tested drugs, slight deviations from the literature data were found (Table 1) and were ascribed to random variations related to the instrument or to electric voltage fluctuations.

It is assumed that the relationship between absorption of light and the properties of the material through which the light is travelling is given by the Beer–Lambert law (Sommer, 1990). In the case of the stationary cuvette, if the detection of the drug is achieved at the same absorbance level as the standard method, then the equivalent detectable concentration level of the same substance using a different optical path length ($l_{\text{stat. cuvette}}$) and/or wavelength ($\lambda_{\text{stat. cuvette}}$) is

$$C_{\text{stat.cuvette}} = \frac{\lambda_{\text{stat.cuvette}}}{\lambda} \cdot \frac{l}{l_{\text{stat.cuvette}}} \cdot C. \quad (1)$$

Now, if the detection wavelength is the same (or only slightly different) for the two absorption methods, the augmentation of the optical path length from 1 to 10 cm yields a 10-fold increase in the corresponding concentration level (Table 2). Thus, extremely low drug concentrations were detected for PenGB in methanol (0.02–0.08 mM), ibuprofen in ethanol (0.09–0.38 mM), hydrocortisone in ethanol (0.0006–0.004 mM) and the *K. brasiliensis* extract solution (0.04–0.4 g/mL). Besides an improvement in the sensitivity of the method (allowing for the detection of small concentrations), the increase in the optical path is also welcome because the calibration curve can now be directly obtained in the large cuvette by spiking aliquots of the standard drug solution.

Linearity

The results of the linearity analysis of the correlation between absorbance and concentration curves of PenGB using both conventional and stationary cuvette methods were assessed in terms of determination coefficient (r^2), as shown in Table 2. The regression equation was calculated by method of least squares for 12 independent assays. It can be observed that the proposed method presented a slight improvement in linearity as compared with the conventional one. Actually, the analytical curve does not need to be linear in the range of application for the method to be efficient. However, in the event that it is, the curve is easier to construct, evaluate and control.

The linear calibration model used was based on the following equation (Skoog *et al*., 1997):

$$y = a + bx + e \quad (2)$$

Such a linear model relates the analyte concentration (x) to the data provided by the analytical instrument (y), where a is the intercept, b the slope and e is a random error assumed to be of zero-mean.

Concerning the concentration range, Beer's law is successful in describing the absorption behaviour of media containing relatively low analyte concentrations. At high analyte concentrations, the average distance between the species responsible for absorption is reduced to the point where each one affects the charge distribution of the neighbours (Skoog *et al*., 1997). The occurrence of this phenomenon instigates deviations from the linear relationship between absorbance and concentration (Skoog *et al*., 1997). In view of this issue, the stationary cuvette method seems to be more appropriate than the conventional method. As it involves the use of low-concentration solutions, Beer's law limitation is reduced.

Table 2. Results of the linearity study using conventional and stationary cuvette methods

Parameter[a]	PenGB		Ibuprofen		Hydrocortisone		*K. brasiliensis* extract	
	Conventional	Stationary cuvette	Conventional	Stationary cuvette	Conventional	Stationary cuvette	Conventional	Stationary cuvette
C (mM)	0.2–0.8	0.02–0.08	0.97–3.89	0.09–0.38	0.01–0.02	0.0006–0.004	4–14[b]	0.04–0.4[b]
n	12	12	12	12	8	8	8	15
b	0.9338	0.9583	0.2047	1.9962	16.6762	164.6156	0.692	0.0498
a	–0.0071	–0.0033	–0.0067	0.0001	–0.0051	0.0067	0.0002	0.1298
R^2	0.9997	1	0.9991	0.9999	0.9989	0.9993	0.9906	0.9975

[a] *C* = concentration range for linear relation, *n* = number of analysed samples, *b* = slope, *a* = intercept, R^2 = determination coefficient.
[b] g/mL.

Table 3. Repeatability and intermediate precision for the stationary cuvette method using PenGB

	PenGB concentration (mM)					
Analyst	Sample 1	Sample 2	Sample 3	Mean	SD	RSD %
1	0.4807	0.4985	0.5054	0.4949	0.0127	2.5753
2	0.478	0.4717	0.497	0.4822	0.0132	2.7312
3	0.4822	0.5101	0.5211	0.5045	0.0201	3.9750
Repeatability	0.4939 ± 0.0112 (2.2580%)					

The determination coefficient (r^2) indicates the percentage of the variance that is explained by the fitted linear mapping of the explanatory variable (ICH, 1996; Skoog *et al*., 1997). For both methods, the determination coefficients were higher than 0.999. It can be concluded that 99.9% of the absorbance variation is ascribed to variability in solution concentrations. According to the r^2 value, a slightly improved adequacy of the model was found for the method developed in this work.

Precision and accuracy

All analytical measurements present some degree of random errors, which must be kept to a minimum in order to achieve an acceptable result with a good confidence level. The accuracy of the method was evaluated by studies of repeatability and intermediate precision. The intra-day assay of repeatability presented a relative standard deviation (RSD%) between 2.6 and 4.0%, and the value of maximum repeatability was 2.3% (Table 3). The intermediate precision was analysed by the *F* test (confidence level $\alpha = 0.05$). No significant differences were observed between the results obtained from different analysts on different days, according to the probe value of 0.94.

The results related to intermediate precision analysis are shown in Table 4. They demonstrate an agreement of nearly 90% between the expected and the measured values. The precision of the method was evaluated by the repeatability, a parameter that evaluates the closeness of agreement in a series of measurements obtained from the same sample using the same instrument and operator. Its value is related to indeterminate and random errors, which cannot be eliminated. The precision can be expressed by the RSD%, which is the relative measurement of the variance and which evaluates how dispersed the data are (ICH, 1996). Since low data variation indicates high precision, the obtained RSD% for the proposed method at a confidence level of 95% attested to its precision.

The intermediate precision was evaluated by means of an inter-laboratory comparison, since the aim of this investigation was the standardisation of an analytical procedure. The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the obtained ones. The analysis requires prior determination of the linearity, the linear interval and the specificity. The accuracy was calculated from the difference between the mean and the accepted true value, considering confidence levels of 95%.

By following the conventional methodology, the confidence range was found to be from 0.0186 to −0.0251. The presence of the zero value within this interval confirmed the nonexistence of systematic errors. This situation was not strictly observed using the stationary cuvette methodology since, for this method, the confidence range was from −0.0053 to −0.0165. Nevertheless, the great proximity to the origin for such an interval and the high value of the r^2 derived in the analysis allow us to consider the absence of systematic errors (ICH, 1996).

Table 4. Accuracy of the stationary cuvette method using PenGB ($n = 9$)

	PenGB concentration (mM)		
Assay	Sample 1	Sample 2	Sample 3
1	0.2452	0.4807	0.6923
2	0.2446	0.4780	0.7059
3	0.2490	0.4822	0.7141
4	0.2537	0.4985	0.7303
5	0.2335	0.4717	0.6943
6	0.2637	0.5101	0.7438
7	0.2297	0.5054	0.7661
8	0.2469	0.4970	0.7307
9	0.2724	0.5711	0.8510
Mean	0.2487	0.4994	0.7365
Theoretical concentration (mM)	0.2503	0.5394	0.7365
Accuracy (%)	99.37	92.58	99.99

Ruggedness

According to the hypothesis test (*t*-Student and Fisher test), no significant variation was observed considering the parameters studied for the ruggedness analysis. As the aim of this work was to develop and to standardise an analytical method, its validity under different conditions (Table 4), such as locality and analysts, should be analysed. Therefore, ruggedness instead of robustness was evaluated. According to the Student *t*-test and ANOVA ($p = 0.05$), no differences were observed between the conventional method and the proposed one. Actually, it was found that they were statistically indistinguishable concerning ruggedness, indicating that the proposed method is also resistant to changes in standard conditions.

Applicability

Table 2 shows the linearity results as well as their comparison with those obtained from the traditional method. Concerning linearity results for ibuprofen, the determination coefficient, the slope and the intercept were found to be 1.0000, 1.9962 and −0.0001, respectively (Table 2). Allowing for hydrocortisone, the

determination coefficient, the slope and the intercept were 0.9994, 164.6156 and 0.0067, respectively (Table 2).

Concerning the *K. brasiliensis* extract, the stationary cuvette technique revealed a higher linearity compared with the conventional method. Therefore, this methodology can easily be used to analyse phytochemical products from plant extracts or pharmaceutics and cosmetic products containing them.

The ruggedness concerning the optical path length was also evaluated. The study using hydrocortisone and ibuprofen evaluated the applicability of the method. The results support the advantages of the developed method, such as straightforward sample preparation, precision and improved linearity and sensibility factors. They also provide evidence that can be applied in the analysis of many other drugs and phytochemical products.

The quality of analytical measurements has always been a subject of the utmost value. Therefore, the development of analytical methods presenting improved calibration parameters and ruggedness is of particular interest. Besides being a rapid procedure and providing high sensitivity, the stationary cuvette method is appropriate to obtain analytical curves by UV–VIS spectrophotometry. The obtained results support the view that the proposed method is accurate, precise, rapid and suitable for drug analysis in raw materials, phytochemical products and medicines. Further advantages of this method include its low cost and low solvent requirements. Both of these properties provide the real versatility of the stationary cuvette methodology for the analysis of pharmaceutical and phytochemical compounds by UV–VIS spectrophotometry. In fact, by using such methodology, the use of reagents may be reduced as much as five times compared with the traditional technique. Concerning the time for analysis, while still comparing with the traditional method, the stationary cuvette procedure requires only 30% of the time. Another important point is the reduction in the number of laboratory materials (such as volumetric flasks, beakers, etc.), which reduces the random errors during the development of the procedure. All of these aspects were proof of the cost reduction of this new method.

Although not an extractive method such as HPLC, spectrophotometry can be quite reliable and presents results with statistical significance compared with the literature (Bunea *et al*., 2008). Therefore, this methodology can be used for those laboratories that do not have available HPLC equipment.

The use of the traditional UV–VIS spectrophotometric method in phytochemical analysis has already been demonstrated in several papers. Grubesic *et al*. (2005) employed this technique to determine total polyphenols and tannins in the *Plantago* species using the Folin–Ciocalteu reagent. They concluded that the method was able to provide new information regarding phytochemical characterisation of these plant species. Similarly, Hubert *et al*. (1999) used spectrophotometry combined with dynamic ultrasonic extraction online. They were able, therefore, to develop a simple, rapid and efficient method for the determination of total flavonoids (calculated against scutellarin) in *S. barbata* Don. The total carotenoid content in fresh, refrigerated and processed spinach (*Spinacia barbata L*.) was also estimated spectrophotometrically for saponified extracts (Bunea *et al*., 2008). Recently, according to Shokrollahi *et al*. (2008), spectrophotometric methods have also been applied to the analysis of trace metals due to advantages such as accuracy, precision, low cost and simple operation. These authors demonstrated that this method could be used effectively to determine trace metals in environmental or industrial applications such as soil and water analysis.

Another important point concerns the versatility of this technique compared with other more modern ones. In fact, although nmR, HPLC, GC, MS and other more expensive and laborious methods that use a combination of such techniques (e.g. TMS; HPLC-PAD-ESI/MS or SPME/GC-MS) can be used, UV–VIS spectrophotometry is still widely employed in routine analytical procedures for active compounds for its simplicity in sample preparation combined with feasibility, specificity, sensitivity and low cost (Hubert *et al*., 1999; Grubesic *et al*., 2005; Shokrollahi *et al*., 2008). Additionally, measurements made by spectrophotometry, although they could be less accurate than those by HPLC, generally gave similar results (Bunea *et al*., 2008; Jin *et al*., 2008). Similarly, although using the traditional spectrophotometric method, Jin *et al*. (2008) developed an assay for total serotonin derivatives in safflower seeds, characterised by simplicity, specificity, sufficient sensitivity and inexpensive instrumentation in comparison with published HPLC analytical methods.

Therefore, the stationary cuvette method may be considered a first-rate alternative to derive analytical curves for analysing drug content in raw materials and medicines as well as in plant extracts through UV–VIS spectrophotometry.

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Trosiéme partie

TRAVAUX EXPERIMENTAUX EN FRANCE

DESIGN AND CHARACTERIZATION OF MICROEMULSION DRUG DELIVERY SYSTEMS

TITLE PAGE

DESIGN AND CHARACTERIZATION OF MICROEMULSION DRUG DELIVERY SYSTEMS

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ABSTRACT

The advantages of microemulsions (ME) over conventional emulsions or others lipid carriers include improved stability and solubilisation properties. In this study, different formulations of MEs were examined to select and characterize an ideal system for parenteral pharmaceutical use. To determine the microemulsion regions, phase diagrams were constructed titrating surfactants/oil mixtures with water at 25°C. Mixtures with compositions corresponding to the different types and structure of microemulsions were analysed by polarized light microscopy, pH, conductivity, rheology behaviour, refractive index, stability evaluation, particle size, and transmission electron microscopy. Among the oils studied, sesame oil yielded the largest ME region. For an oil with a small ME region (Miglyol 812), an increase in the ME area was observed when a Pluronic surfactant type is used. The rheological study revealed that most systems showed a non linear relationship between the shear stress and shear rate, thus exhibiting non-Newtonian flow properties. All the MEs studied in this work become turbid after their dilution but the nanoemulsions so formed remained stable for at least 7 days at room temperature. Notably, all the formulations studied were free of alcohols and all components were drug-grade agents, assuring that the systems could be safely used in the clinic.

Key words: Microemulsions systems, internal microstructure, characterization

Introduction

More than 50 years ago, pharmaceutical technology could only offer two therapeutically and commercially viable dispersed systems: emulsions and the suspensions [1]. Now, with the fast advance in the development of new equipment and processes, emulsions, originally defined as simple mixtures of water and oil stabilized by a surfactant, have become a group of “emulsion systems”. This group includes all the systems formed from a mixture of water, oil and surfactant in specific proportions: ordinary emulsions or macro emulsions; submicronic emulsions, also known as nanoemulsions [2]; microemulsions (MEs), liquid-crystalline systems, and also systems classified as self-emulsifying (nano, micro or macro) [3-6]. Although there are some variations in this classification, scientific articles concerning these system appear in the literature every day, patents are registered and products based on these systems are being launched [7].

Another concept that has evolved over the same period is nanotechnology. It currently occupies a special space in the pharmaceutical area, especially with respect to the development of new drug delivery systems, which provide many advantages compared to traditional systems, including their versatility. These two concepts have come together to stimulate research towards the development of new emulsified systems, particularly those on the nanometric scale.

This work concentrates on MEs as described by Danielson and Lindman[8]: thermodynamically stable dispersions of oil and water that are stabilized by surfactants and in some cases, additionally by co-surfactants. MEs have attracted much interest in recent years because of their great practical importance in terms of drug delivery potential and interesting physical properties. Excellent reviews can be found in the literature that describe both the physical properties and the pharmaceutical and cosmetic application of ME systems [2, 9-14].

Since the development of MEs is an empirical process, the first step is often to construct Pseudo Ternary Phase Diagrams (PTPD) from previously chosen ingredients [4, 15-22]. First, the ME regions are determined and, thereafter, the influence of substituting some of the components on the formation of certain microstructures can be investigated [5, 23]. However, there are still divergences in the literature concerning the choice of parameters and tests to identify a mixture of oil, water and surfactant as a ME. While MEs can be prepared very easily, it is far from trivial to characterize their microstructure [24, 25]. As a result of the low interfacial tension between oil and water, a wide range of ME structures is possible. Basically two basic types of MEs can be distinguished: bicontinuous and droplet structures. In a bicontinuous structure both water and oil form continuous domains separated by surfactant-rich interfaces [26]. They are likely to occur when similar amounts of oil and water are used; otherwise, droplet structures are formed [27]. At low water content, rod-like micelles are observed, but water-in-oil (W/O) spherical droplets, are also present, as in classical emulsions. In the water-rich region, O/W droplets are the most frequent form. Droplets begin to interconnect with several “bridges”, described as the percolation phenomenon [23, 28]. The tendency toward a water-in-oil or an oil-in-water ME depends on the properties of the oil and the surfactant [29].

The type of ME structure has a strong influence on the rate of drug release and it is possible to tailor the ME type to provide the desired release profile for the solubilised drug [30]. In an O/W ME, hydrophobic drugs, solubilised mainly in the oil droplets, are hindered in their diffusion and are therefore released rather slowly (depending on the oil/water partitioning). The diffusion of water-soluble drugs, on the other hand, is less restrained and they are rapidly released. The reverse behaviour would be expected in W/O ME type [12, 22, 23, 31-34].

The special features of MEs confer on them several advantages for pharmaceutical use, such as ease of preparation, long-term stability, a high solubilisation capacity for both hydrophilic and lipophilic drugs, improved drug delivery [17, 35-39], leading to a wide range

of potential applications. MEs can be used for topical, oral, parenteral and ocular drug delivery. It is important to characterize ME in detail because the behavior of the delivery of the drug incorporated into the system depends on the internal microstructure [17, 40]. However, appropriate physicochemical characterization of ME formulations is challenging because of their small particle sizes and labile interfaces. Therefore, it is necessary to use combination of different characterization techniques [23, 25, 41-45].

In this study, several properties of MEs were examined to select and characterize a ME system suitable for parenteral pharmaceutical use. Several PTPD were produced, using different lipophilic phases and surfactant systems.

Materials and Methods

Materials

Sesame and sunflower oil, Miglyol 812 and Miglyol 829 were the oil phases chosen for this study. They are included in the FDA Inactive Ingredients Database, and in the additives licensed in the UK for use of the intramuscular (IM) and subcutaneous (SC) injections, oral capsules, emulsions, tablets and topical preparations [46].

The surfactants used in this study were Tween 20® (Polysorbate 20) and Span 80® (sorbintan oleate), obtained from Vetec Química fina Ltda, Brazil; Epikuron 200 (lecithin), obtained from Lucas Meyer Co.; Cremophor EL (polyoxyl 35 castor oil), obtained from BASF Aktiengesellschaft, Germany; Imwitor, obtained from Sasol Germany GmbH; and Labrafil M1944 (oleoyl polyoxylglycerides), obtained from Gattefossé, St. Priest, France. All of these are specified in the American and European Pharmacopeias, and also in the Handbook of Pharmaceutical Excipients as suitable for use in cosmetics preparations [46].

Methods

Two systems were chosen to construct PTPD. The first one contained Miglyol 812 as a lipophilic phase and different mixtures of surfactants, while the second used fixed amounts of Tween and Span and varied the other phases. Phase diagrams were created by visual inspection of the surfactant mixtures. The oil phase was added in the proportions from 1:9 to 9:1 with respect to the surfactants. The aqueous phase was titrated and stirred at 25ºC to reach equilibrium. The mixtures were checked both visually and by polarized optical microscopy. The ME phase was identified as the region of the PTPD where clear and transparent formulations are obtained, based on visual inspection. Its isotropy and thus non-birefringent behaviour was confirmed by examination under polarizing light.

Sample preparation: The samples were prepared by combining appropriate amounts of each component followed by magnetic stirring for 5 min. ME compositions are given in Table 1, corresponding to the ME region of each PTPD.

Table 1. Composition of the microemulsions samples studied

Sample	Oil Phase (%)	Surfactants (%)		Aqueous Phase (%)
	miglyol 812	**Epykuron 200**	**Cremophor EL**	**Water**
1.1	15	37,5	37,5	10
1.2	30	30	30	10
1.3	40	25	25	10
1.4	50	20	20	10
1.5	40	20	20	20
1.6	40	15	15	30
	Miglyol 812	**Tween 20**	**Span 80**	**Water**
2.1	30	32,5	32,5	5
2.2	38	28,5	28,5	5
2.3	48	23,5	23,5	5
	Sunflower oil	**Tween 20**	**Span 80**	**Water**
3.1	20	37	37	6
3.2	30	32	32	6
3.3	40	27	27	6
	Sesame oil	**Tween 20**	**Span 80**	**Water**
4.1	5	54	36	5
4.2	10	48	32	10
4.3	20	36	24	20
4.4	20	45	30	5
4.5	20	42	28	10
4.6	30	36	24	10
4.7	40	30	20	10

Refractive Index: The refractive indices of the samples were measured at 25ºC with an Abbe Refractometer (ANALYTIKJENA AG) with a Thermo Haake C10 heating system.

Polarized optical microscopy: A drop of sample was placed between a cover slip and a glass slide and examined under polarized light. A microscope equipped with camera (LEITZ WETZLAR, Germany) was used to examine the various fields of the phase diagram at room temperature and to verify the isotropic behaviour of the ME.

Conductivity: The conductance was determined at room temperature using a Digimed conductimeter (Modelo DM-32), São Paulo/ Brazil.

pH: The pH was determined at room temperature using a pHmeter Tecnal TEC-2 Model, São Paulo/ Brazil.

Rheology: The influence of the type and amount of oil and surfactant on the internal structure of the ME can be studied by rheological measurements. The rheological parameters were measured using a rotational rheometer Rheostress 600 (Haake, Karlsruhe, Germany). Measurements were performed with a stainless steel cone/plate measurement device of 35 mm in diameter, a cone angle of 2° and a gap of 105 µm. The sample compartment was maintained at 25ºC using a Haake DC-30 water bath/circulator and a Haake Universal Temperature Controller system (UTC) (Haake, Karlsruhe, Germany).

Particle size measurement: MEs were diluted in water to 5 mL and gently mixed by inversion of the flask. Droplet size distributions of the systems were determined by dynamic light scattering (Zetasizer NanoZS,Malvern Instruments, Malvern, UK) at room temperature. The instrument contains a 4mW He-Ne laser operating at a wavelength of 633 nm. The measurements were made at a detection angle of 173º.

Transmission Electronic Microscopy (TEM): For observation by TEM, a drop of ME was placed on a cooper grid. Before analysis, the MEs were stained by a 1% phosphotungstic acid aqueous solution. TEM analysis was performed using a Philips EM208 (1996) instrument equipped with a wide-field CCD camera.

Stability evaluation

Shelf-life: ME stability was routinely evaluated as a function of storage time by visual inspection of the samples initially on a daily and later on a month basis. Stable systems were identified as those free of any physical change such as phase separation, flocculation or precipitation.

Freeze-Thaw Cycle: Samples were submitted to freeze-thaw cycles (-20 to 20 ºC) of 24 h for a seven-day period. Their physical stability was assessed by the criteria described above.

Centrifugation: The samples were centrifuged at 10000g for 30 min (Jouan MR 1812,) After centrifugation, their macroscopic appearance was observed and the particle size was measured.

Dilution test: The aim of this test was to determine whether these systems could be diluted with the external phase of the systems without phase separation. Selected MEs were diluted 1:10 in water and were observed by polarized optical microscopy for optical isotropy. Systems were considered as true MEs when their physical integrity was preserved after dilution.

Results and Discussion

ME formation depends on two factors: the properties of oil and surfactants and the ratio of oil and water. Zwitterionic surfactants are often used for pharmaceutical applications because they are less toxic and less sensitive to pH and ionic strength changes [5, 15, 47].

Analysis of refined sesame and sunflower oil indicates their composition in fatty acids, present a mixture of long-chains: arachidic acid, linoleic acid, oleic acid, palmitic acid and stearic acid. These oils are extensively used in cosmetics and pharmaceutical formulations. They have been shown to be safe for use in intramuscular injections without inducing tissue damage and are generally regarded as nontoxic and nonirritant [46] . Miglyol neutral oils consist of a mixture of triglycerides of saturated fatty acids, mainly of caprylic and capric acid. These medium-chain triglyceride oils are widely used in pharmaceutical formulations because of their stability, biocompatibility and their ability to solubilise drugs.

The PTPD presented in Figure 1 show the influence of the surfactants, oil and aqueous phase on the ME region. In particular, they highlight the influence of the nature of the surfactants on the phase properties. Clear, isotropic and one-phase systems were classified as ME while systems showing birefringence with characteristic oily steaks, Maltese crosses or textures were designated as systems containing a lamellar mesophase (LM). Non-birefringent two-phase systems as determined by visual inspection and microscopy were designated as coarse emulsion.

Sesame oil showed a larger ME region than the other oils studied when Tween 20 and Span 80 were used as the surfactant system (Figures 1A, 1B, 1C, 1G). No significant differences were observed between the phase diagrams obtained with Miglyol 812 and Miglyol 829 with the same surfactant system (compare Figures 1C and 1G). When Miglyol 812 was used as the lipophilic phase, a larger ME region was obtained when Epikuron 200/ Cremophor EL was used as the surfactant system (Figure 1D) or when a Pluronic surfactant was added to the aqueous phase (Figure 1I). Replacing Span 80 by Imwitor did not

significantly influence the ME region (Figure 1E). When Labrafil was used with Tween, only conventional emulsions were formed (Figure 1H); however an ME region appeared when Epikuron 200 was mixed with Labrafil (Figure 1F). This is probably due to the capacity of Epikuron 200 to form bilayers that could be organized in the form of ME in presence of Labrafil.

Isotropic and anisotropic materials can be distinguished by polarized light microscopy, and this can give information about the structure and compositions of materials as ME. Since the isotropic materials have only one refractive index and do not restrict the passage of light in any the vibrational direction, therefore, they exhibit the same optical properties in all directions. On the other hand, the optical properties of anisotropic materials change with the orientation of the incident light because the different crystallographic axes of the material have different refractive indices [18, 20, 45]. All the analysed samples that demonstrated isotropic behaviour on analysis by polarized light microscopy (Table 2) obey the criteria of Danielson and Lindman [26] and can be considered as MEs. However, some systems containing Epikuron and Cremophor as the surfactant system (series 1) showed anisotropic properties. The values of the refraction index close to 1.4 measured for the isotropic samples (Table 3) confirm the transparency of the systems and indicate that the ME would be suitable for ophthalmic formulations.

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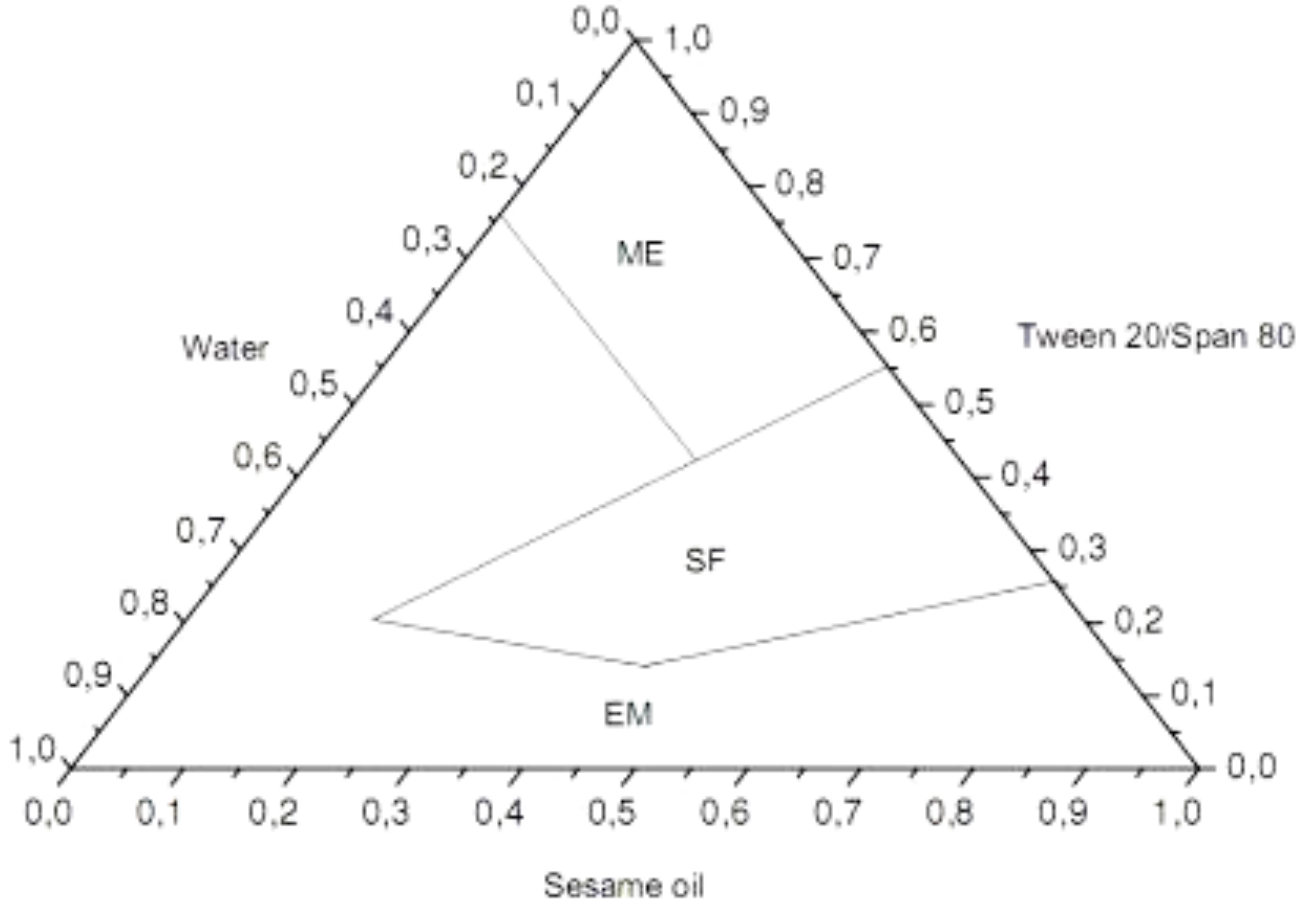
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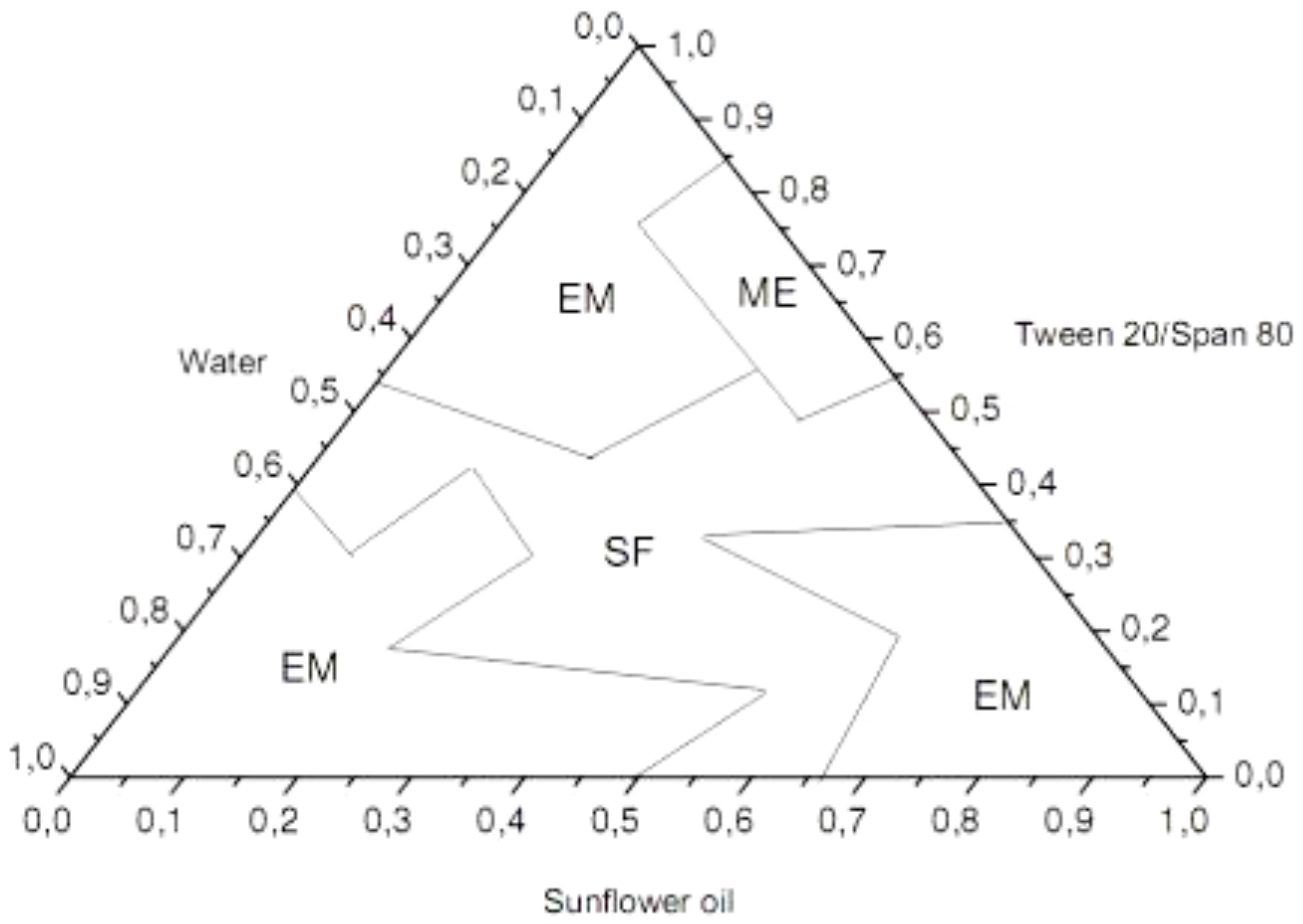
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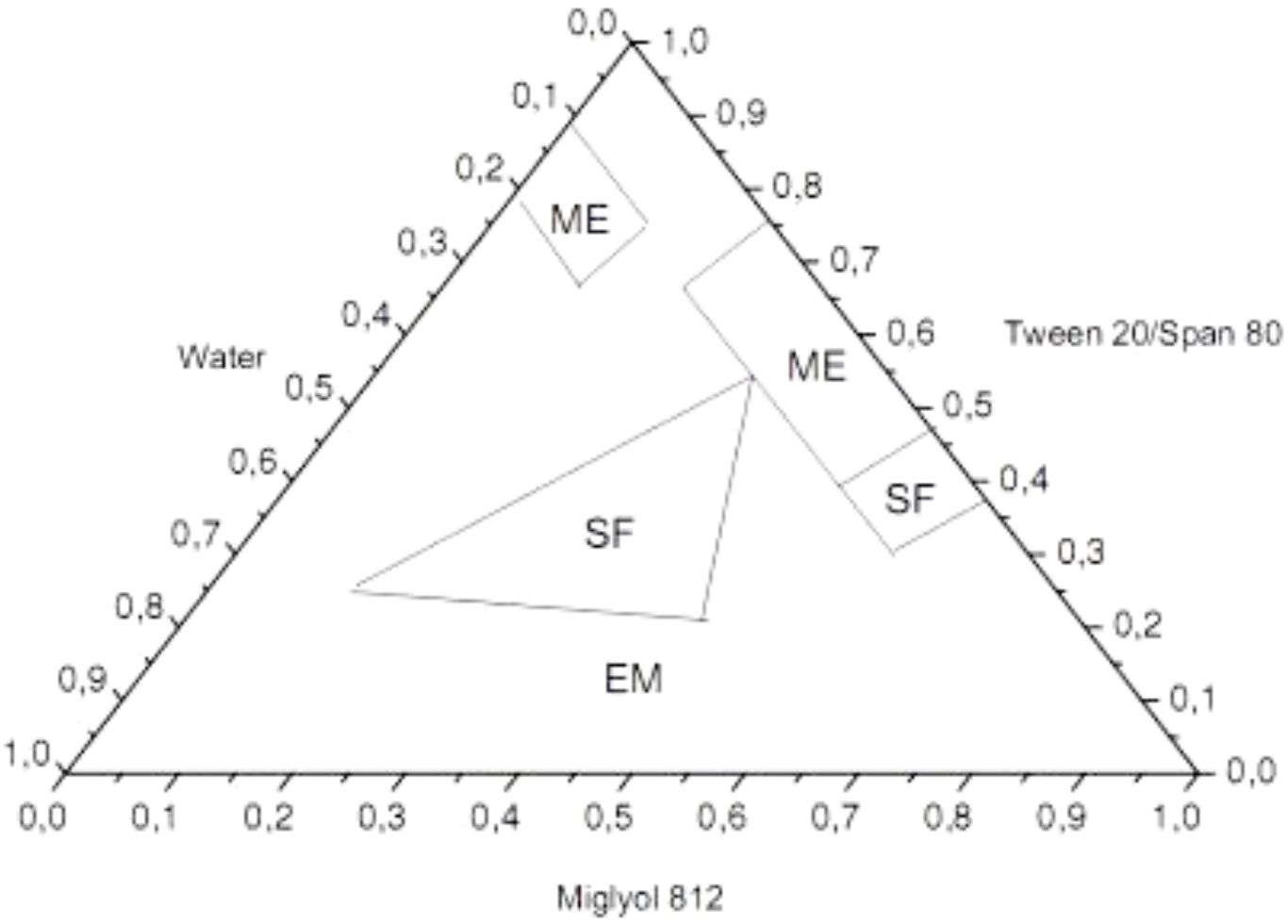
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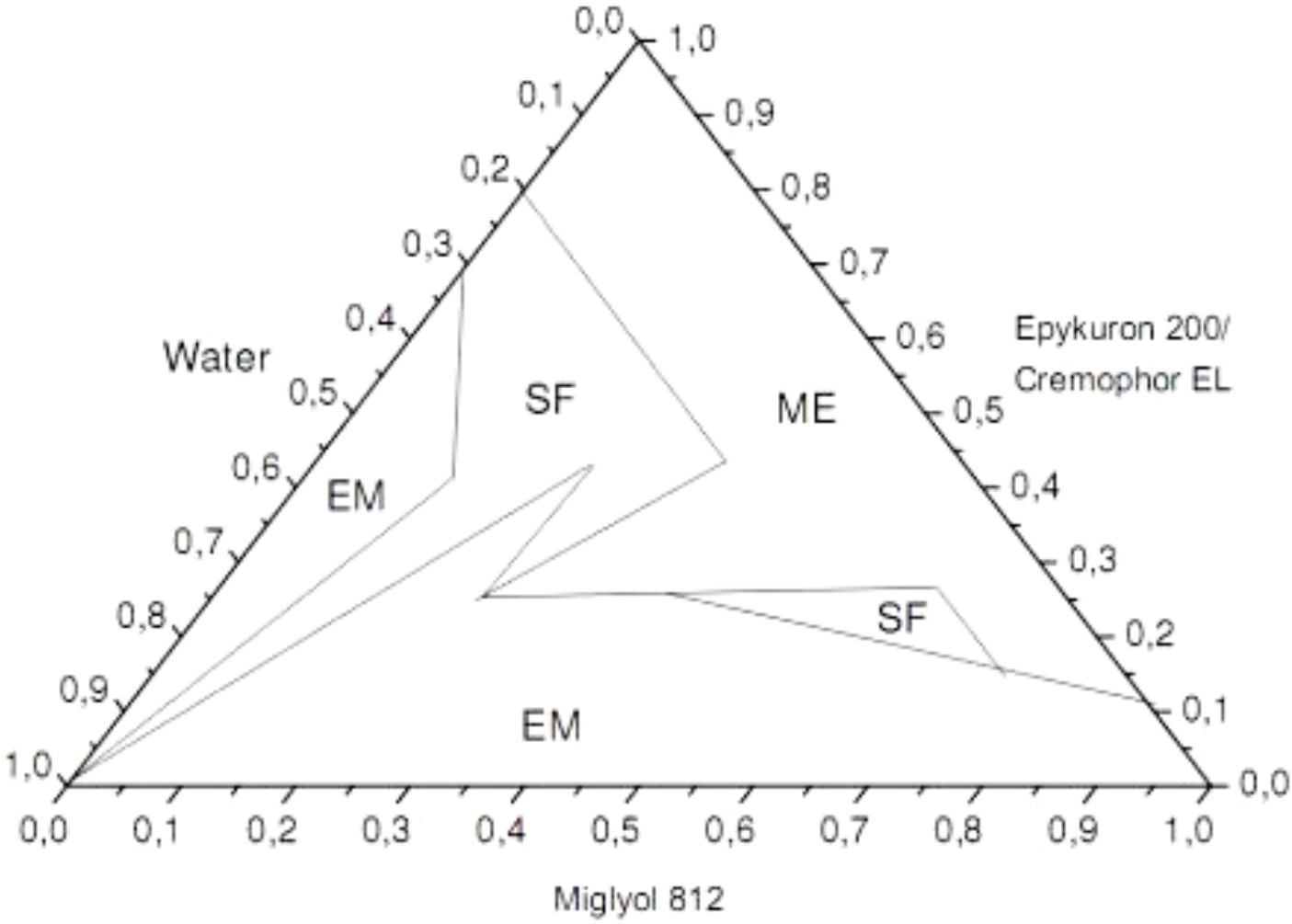
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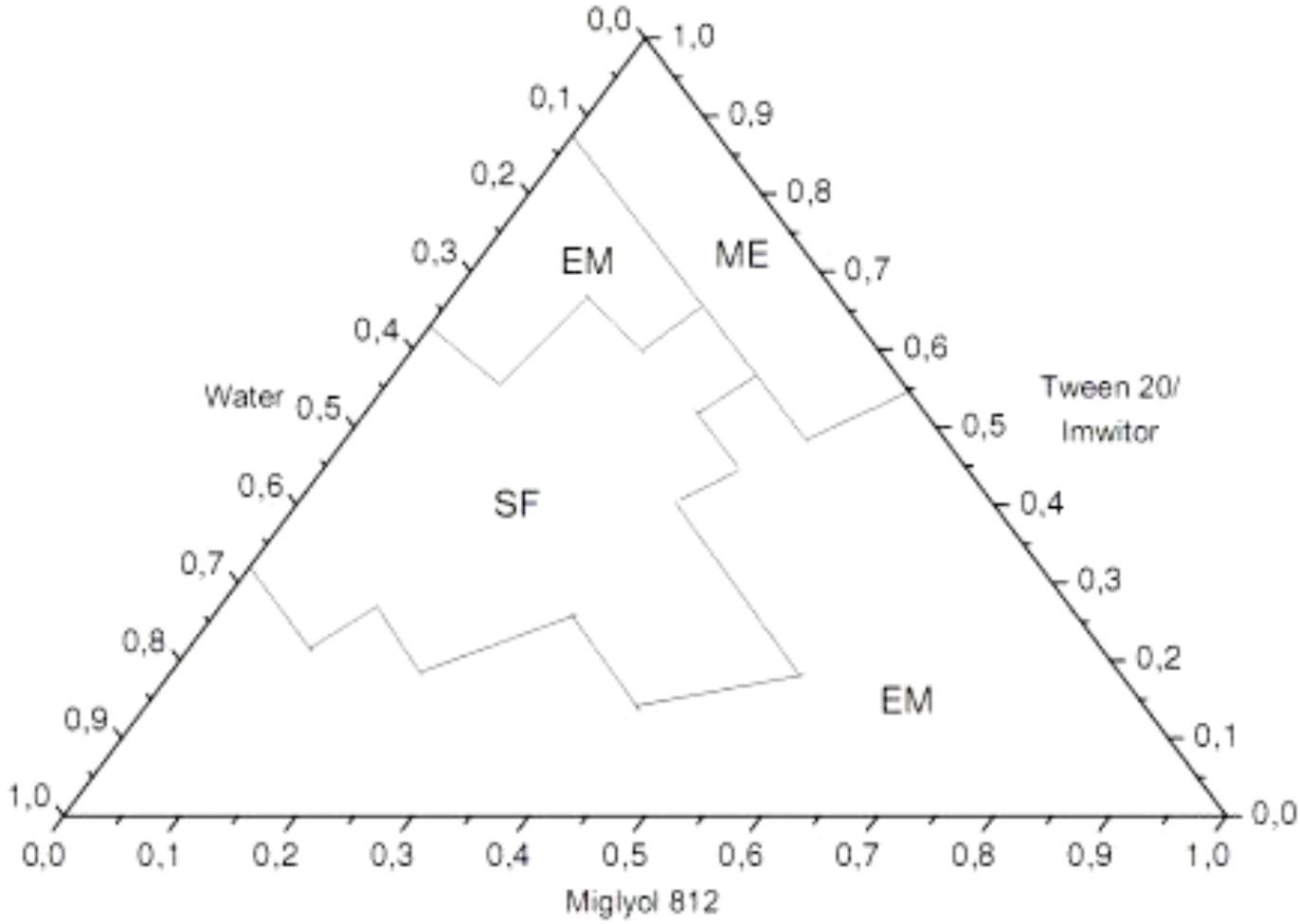
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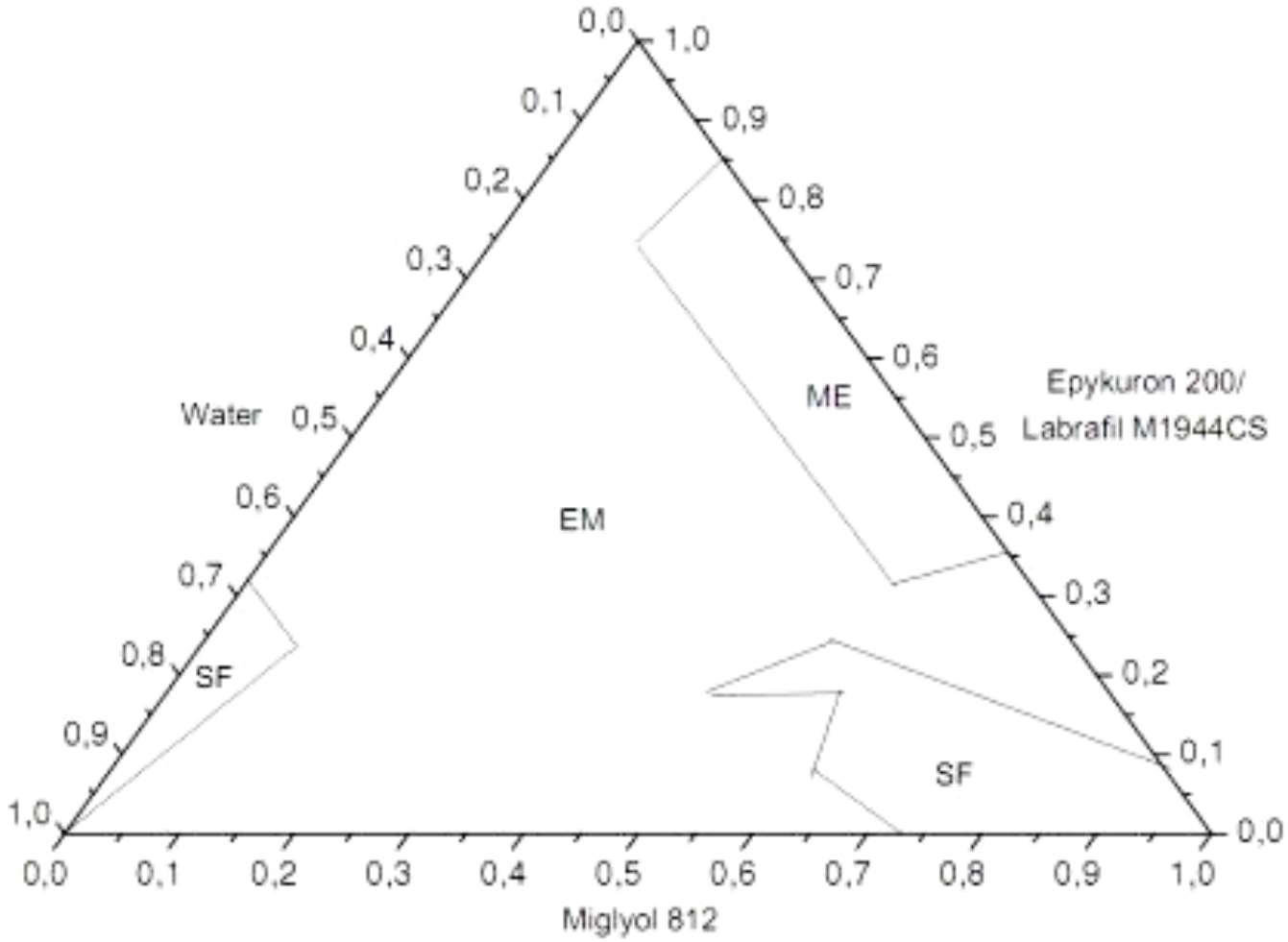
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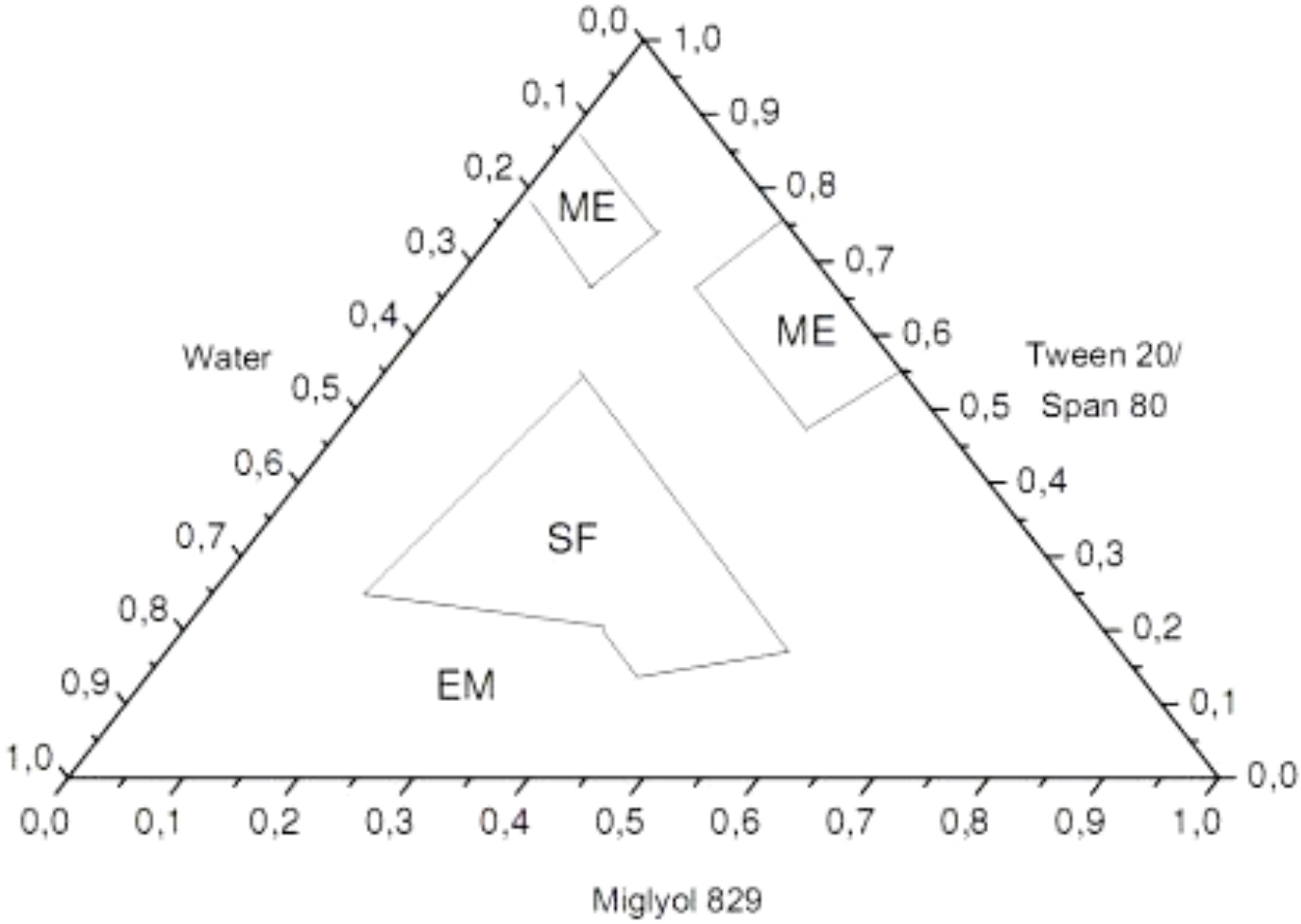
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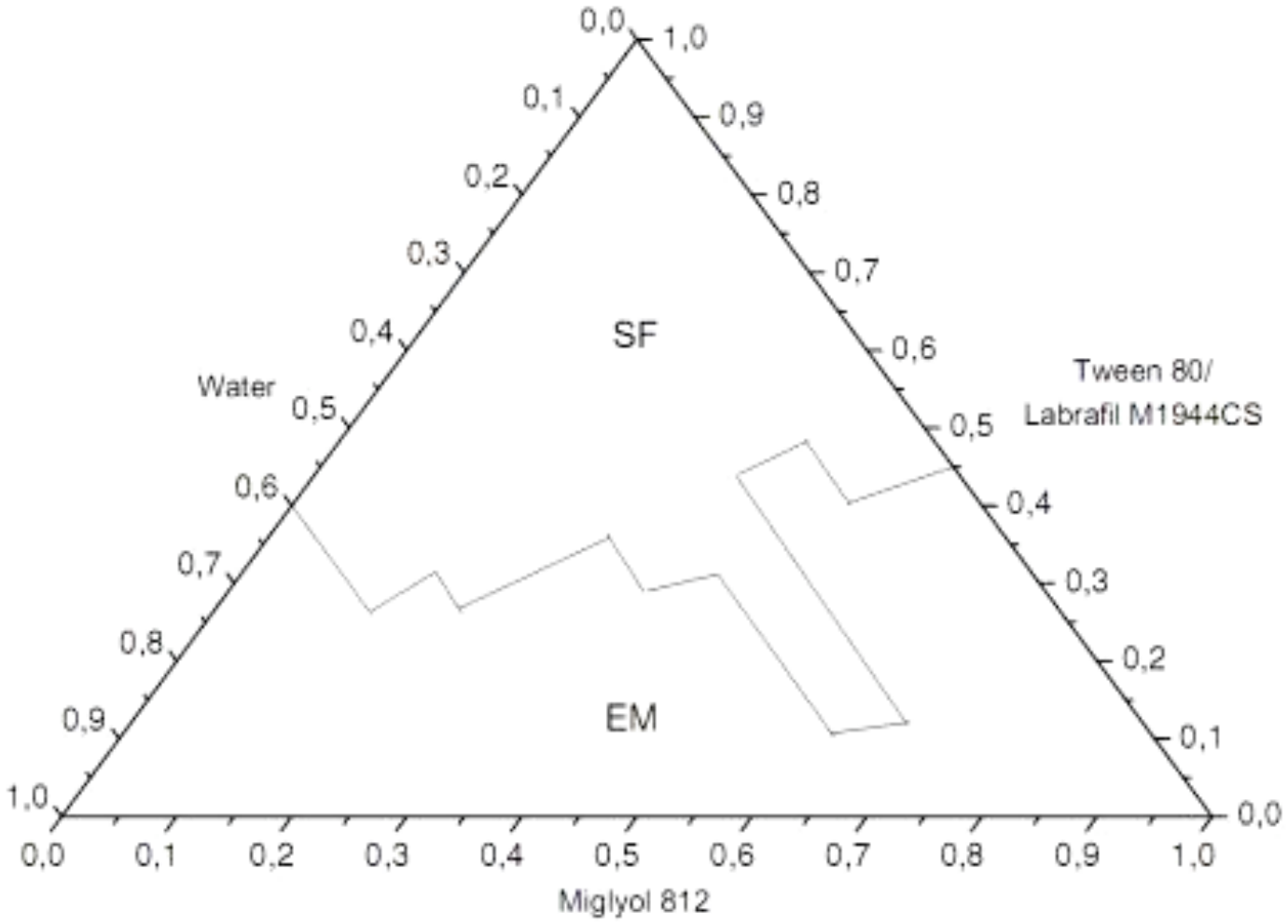
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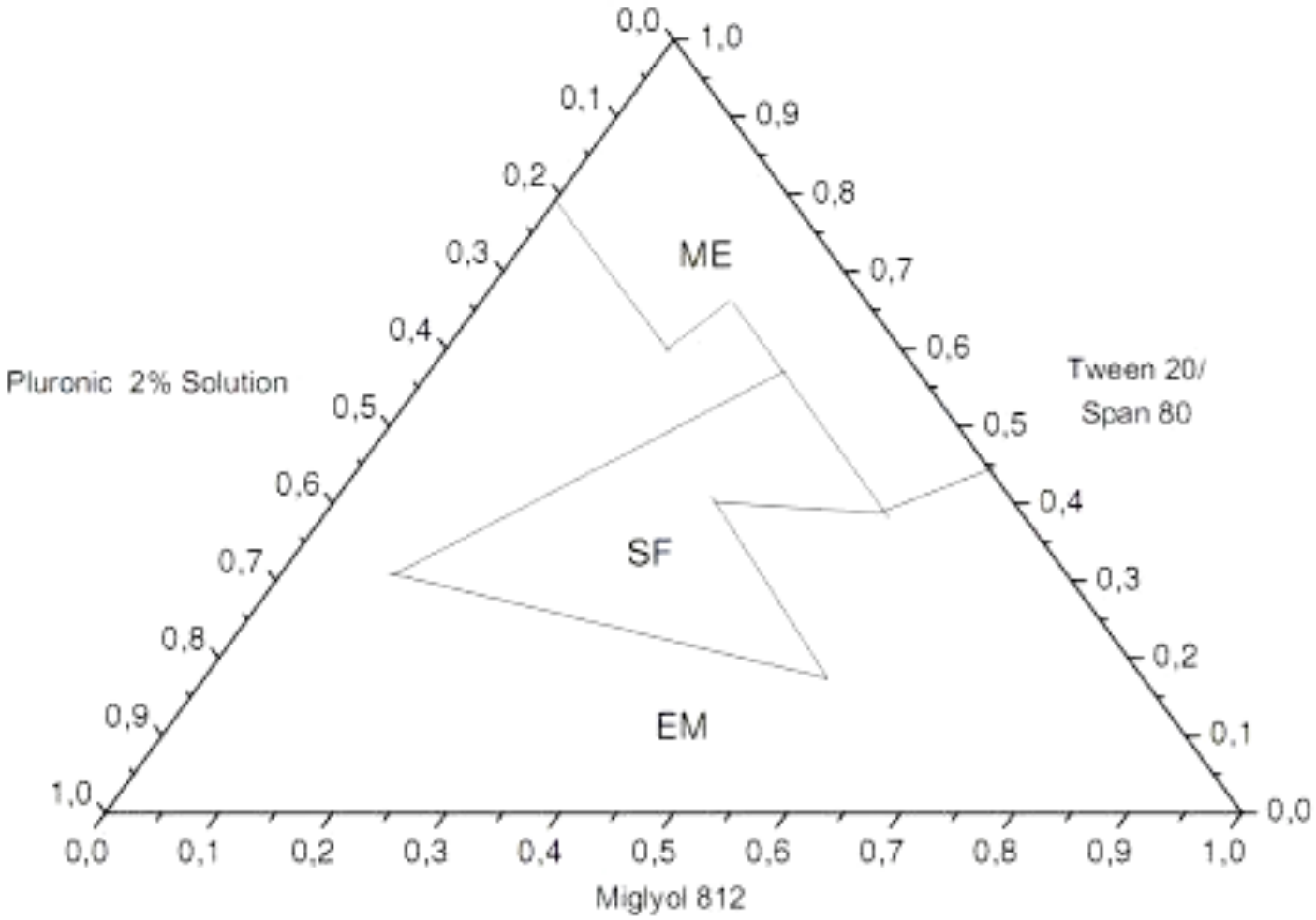

Figure 1. Pseudoternary phase diagrams

Although the determination of pH does not give information about ME structure, this information can guide the choice of possible drugs to be incorporated into these systems. For example, Junyaprasert [12] showed that the incorporation of local anesthetics into Brij 97-based MEs can be affected by their pH [5, 15, 47]. All the microemulsions examined in this study had pH values close to neutrality (Table 3) and so would be suitable for incorporating molecules that are sensitive to extremes of pH.

Table 2. Rheological Behavior and viscosity results of the Sesame oil microemulsion.

Sample	Rheological Behavior	Viscosity (Pa)
4.1	Pseudoplastic	0,704
4.2	Pseudoplastic	0,691
4.4	Pseudoplastic	0,547
4.5	Pseudoplastic	0,45
4.6	Pseudoplastic	0,32

Studies of rheological behaviour studies during the development of emulsion systems can be a very important tool to assess their stability [48]. The most useful rheological profile is one which shows the shear stress (τ) as a function of the shear rate ($\hat{y}$). The flow curves obtained for most emulsion systems show a non linear relationship between the shear stress and shear rate, indicating non-Newtonian flow properties. The colloidal structure breaks down when the shear rate is increased, and the viscosity is reduced. Figure 4 demonstrates that the samples containing sesame oil showed a pseudoplastic flow, with reduced viscosity as the shear stress increased. This type of behavior is desirable in pharmaceutical formulations because it is necessary to have an apparent elevated viscosity at low shear rates to avoid mobility of the dispersed phase. Moreover, it is important that the product flows freely when stirred, presenting low viscosity at high shear rates; however, this change must be reversible to retard coalescence or creaming of the product [49-52]. Measurement of the viscosity helps to determine whether a product presents the appropriate consistence or fluidity and can predict the stability of the product over time. Samples 4.1, 4.2, 4.3, 4.4 and 4.5 had relatively constant values at 0.3 to 0.8 Pa. Table 4 shows the apparent

viscosity of the Sesame oil formulation at a tension of 2.5 Pa. It can be noted that the viscosity values tend to increase slightly with the increase of the surfactant concentration what might be the result of interaction between the surfactant molecules.

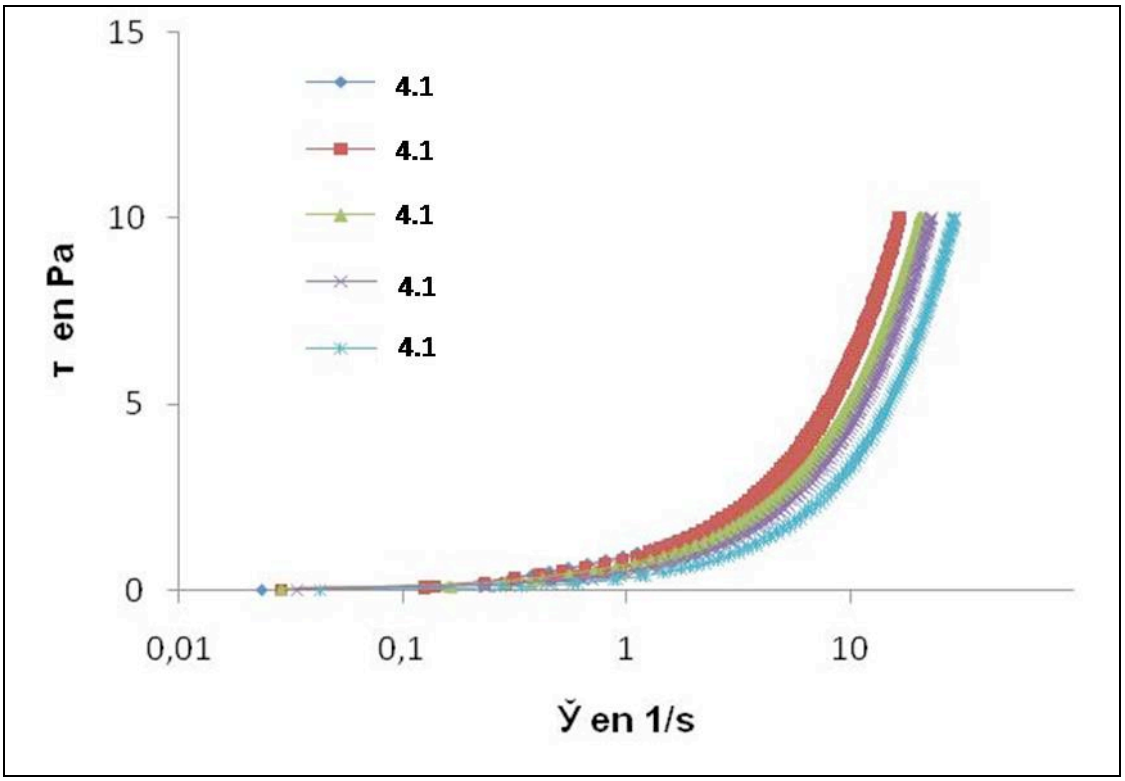

Figure 2. Rheological behavior of the Sesame oil microemulsions.

Conductivity measurements can help to determine both the nature of the continuous phase of a ME and the percolation threshold, at which the droplets begin to interconnect and transform the ME to a bicontinuous type [22, 26]. This transformation also brings about changes in the viscosity. Thus, an increase of the conductivity and the viscosity in samples at the same concentration of water indicates that the system changes from one of isolated droplets to an interconnected bicontinuous structure, suggesting a possible phenomenon of percolation [53, 54]. The clustering of the droplets at the percolation threshold typically leads to an increase in both viscosity and conductivity [29, 55].

Table 3. Results of the pH, refractive index, conductivity and size analysis of the microemulsions samples.

Sample	pH	IR	Conductivty	Size	PdI
2.1	7,85	1,46	0,73	187,0	0,187
2.2	7,05	1,46	0,60	183,9	0,208
2.3	7,55	1,46	0,63	170,0	0,198
3.1	6,93	1,47	1,35	199,6	0,247
3.2	7,08	1,47	1,20	197,7	0,288
3.3	6,87	1,47	1,09	167,8	0,256
4.1	6,89	1,47	1,46	200,3	0,378
4.2	7,27	1,46	2,84	273,3	0,425
4.4	7,42	1,47	1,32	233,5	0,367
4.5	7,28	1,46	3,41	278,0	0,415
4.6	7,27	1,46	4,12	294,2	0,428

Quasi-elastic light scattering is a routine technique for the determination of the diameter of colloidal particles and has been applied to the analysis of the internal phase of MEs. Although this technique is not able to distinguish between droplet–type and bicontinuous structures [15], it is useful complement for characterisation Such measurements were carried out for the liquid MEs after dilution in water or aqueous buffer in order to detect experimental errors. The diameter and polydispersity index (PDI) of the droplets are shown in the Table 3. [10, 56].

Microscopic analysis of for sensitive structures such as MEs is delicate because the structure can easily be altered by the preparation of the samples. TEM analysis is an appropriate technique because the sample is simply deposited on a copper grid. A typical image of the morphology of MEs by TEM analysis is shown in Figure 2. ME show a spherical shape and uniform droplet size, and of droplet size confirm the results obtained by DLS.

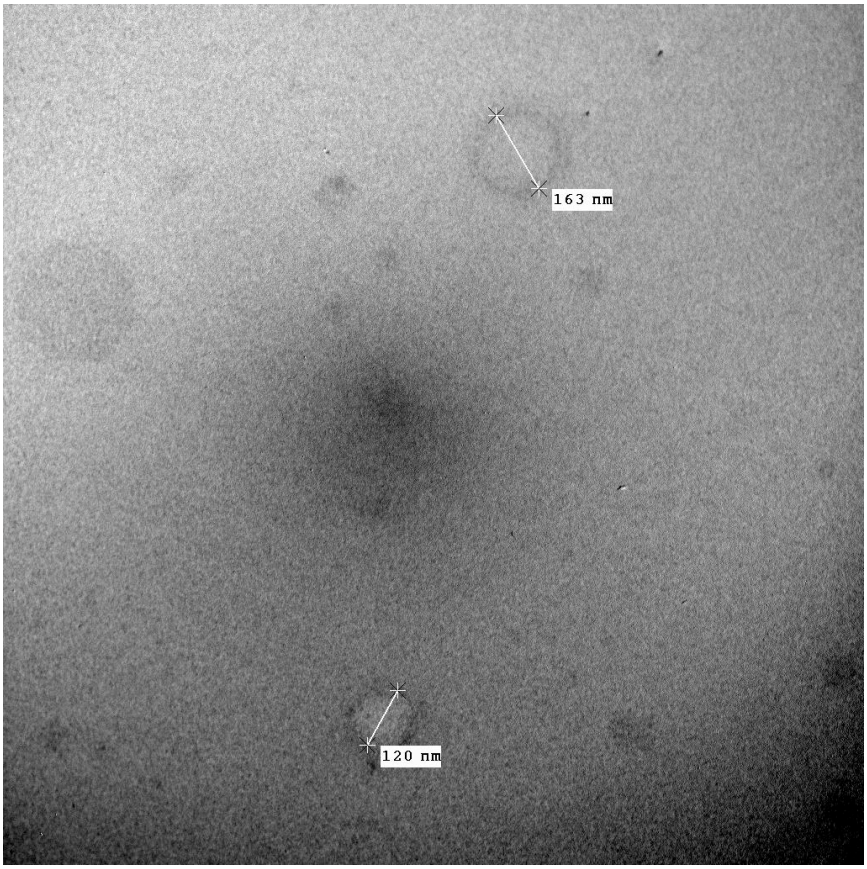

Figure 3. Transmission electronic microscopy photography of the 3.3. sample.

The stability results are shown in the Table 2. As can be observed, MEs remained stable throughout the study under almost all conditions except for the containing Lecitin and Cremophor. All the MEs studied in this work became turbid after dilution but the nanoemulsions formed remained stable for at least 7 days at room temperature. The results are in strong contrast to the systems described in Moreno's work [57] Such differences could be explained by the interfacial activities of the surfactants used to obtain the ME systems. According to Moreno, true MEs are those that preserve their isotropic and transparency characteristics after dilution. If this criterion is taken into account, the systems prepared in this work must be classified as self-nanoemulsifying systems, because they allow the formation of stable nanoemulsions immediately after their dilution with minimum stirring. Nevertheless, these systems fulfill all the Danielson and Lindman [26] criteria regarding isotropy, transparency and stability.

Table 2. Results from aspect analysis, diluition test and stability evaluation of microemulsions systems.

Sample	Aspect		Diluition test (1 :10)	Stability Test		
	Microscopic	Macroscopic		Centrifugation	Shelf-life	Freeze-traw
1.1	Anisotropic	Winsor IV	Nanoemulsion	Instable	Stable	Instable
1.2	Anisotropic	Winsor IV	Nanoemulsion	Instable	Stable	Instable
1.3	Anisotropic	Winsor IV	Nanoemulsion	Instable	Stable	Instable
1.4	Anisotropic	Winsor IV	Nanoemulsion	Instable	Stable	Instable
1.5	Anisotropic	Winsor IV	Nanoemulsion	Instable	Stable	Instable
1.6	Anisotropic	Winsor IV	Nanoemulsion	Instable	Stable	Instable
2.1	Isotropic	Winsor IV	Nanoemulsion	Instable	Stable	Instable
2.2	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
2.3	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
3.1	Isotropic	Winsor IV	Nanoemulsion	Instable	Stable	nstable
3.2	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
3.3	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
4.1	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
4.2	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
4.3	Anisotropic	Winsor IV	Nanoemulsion	Instable	Instable	instable
4.4	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
4.5	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
4.6	Isotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable
4,7	Anisotropic	Winsor IV	Nanoemulsion	Stable	Stable	Stable

Conclusions

A systematic structural study of microemulsion samples prepared with different surfactant and oil contents was carried out by photon correlation spectroscopy, rheological behaviour, polarized light microscopy with the aim of establishing relationships between the structural aspects of the so-called MEs and their composition. Although the drug delivery potential of MEs is widely accepted, the mechanism by which drugs are incorporated and released is not yet well understood. This is probably due to a variety of factors depending on the composition and the resulting microstructure of the formulation. Therefore, it is important to know the internal structure of a ME and in particular whether it presents as dispersed droplets or as a bicontinuous structure. To obtain this refined structural picture of the ME, a combination of various techniques must be useful in connection with an appropriate theoretical model.

The biocompatibility of the system must also be considered. The compositions which give rise to stable MEs generally include medium chain length alcohols as co-surfactants. However, most alcohols are harmful to the human body, so the ME-based drug delivery systems may cause irritation or toxicity. Therefore, it should be stressed that all the systems described in this study are free of alcohols and contain only components that are drug-grade. The high solvent to surfactant volume ratio of these MEs suggests that these systems are microstructured with segregation into oil and water domains and with monomolecular layers of surfactants molecules separating the domains.

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INFLUENCE OF PREPARATION METHOD ON THE STABILITY OF PENICILLIN G BENZATHINE NANOEMULSION

TITLE PAGE

INFLUENCE OF PREPARATION METHOD ON THE STABILITY OF PENICILLIN G BENZATHINE NANOEMULSION

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ABSTRACT

The aim of this work was to study the influence of different methods of preparation of O/W nanoemulsions for parenteral administration of Penicillin G Benzathine (PenGB) on their droplet size and stability. Three methods and their influence on the nanoemulsion properties were evaluated. The investigated methods were high-energy emulsification (US), high-pressure homogenization (UT) and low-energy spontaneous emulsification (SE). All formulations were evaluated for their physicochemical characteristics and parameters for 30 days. Droplet size was measured using a dynamic light scattering and the stability study was carried out by a Turbiscan Analyzer. After preparation, all formulations presented a milky aspect. From the conductivity results (5-50µS/cm), it was observed that all nanoemulsions were O/W type. The smallest mean particle size and polydispersity index (PdI) were found for the SE nanoemulsions. The Turbiscan analysis reveals that nanoemulsions prepared with SE and US presented best stability after one month. On the other hand, UT nanoemulsions showed instability from 10 days. US and UT systems were not able to correctly entrap and retain PenGB and the SE method gave the best results with all the added PenGB entrapped. The stability of this chosen formulation will be further assessed and will be optimized by sequential factorial designs. These results show that the properties of these systems depend not only on their composition but also on their preparation method.

Introduction

The determining factor for the efficacy of β-lactam antibiotics such as PenGB is the time for which drug concentrations exceed the minimum inhibitory concentration (MIC) for the specific pathogen at the site of infection. Severe infections such as endocarditis and osteomyelitis caused by penicillin-susceptible organisms such as *Enterococcus* and *Streptococcus* species, which generally require high-dose parenteral therapy for prolonged periods, may benefit from β -lactam therapy [1] . Intermittent-dose intravenous penicillin therapy is impracticable outside the hospital; however the development of nanosystems encapsulating PenGB capable of providing sustained release may be a practical alternative for the treatment of some infections caused by susceptible pathogens.

The development of biocompatible and biodegradable drug carriers with a small particle size, high loading capacity, extended circulation time, and ability to accumulate at the appropriate pathological sites in the body, for the delivery of poorly soluble pharmaceuticals still has many unresolved issues [2]. The availability of such carriers would be highly desirable since the therapeutic application of hydrophobic, poorly water-soluble agents presents some serious problems [3]. Firstly, low water-solubility results in poor absorption and low bioavailability, especially after oral administration. Secondly, the aggregation of poorly soluble drugs upon intravenous administration might lead to complications including embolism, resulting in side effects as severe as respiratory system failure, and can also lead to high local drug concentrations at the sites of aggregate deposition, provoking local toxic effects of the drug and lower systemic bioavailability [4-6].

To overcome the poor solubility of some drugs certain clinically acceptable organic solvents, and/or surfactants are used in formulations. Salt formation or pH adjustment can facilitate the dissolution of poorly soluble drugs in some cases if they contain ionisable groups [6]. More recent approaches include the use of liposomes [7], microemulsions [8],

nanoemulsions [9], nanocapsules [10] and cyclodextrins [11] to increase the bioavailability of poorly soluble drugs [5, 6, 12].

Nanoemulsions are only kinetically stable. However, long-term physical stability of nanoemulsions (with no apparent flocculation or coalescence) makes them unique and they are sometimes referred to as “approaching thermodynamic stability” [13]. Since nanoemulsions have great potential of in pharmaceutical products, an intensive study was performed to determine the influence of the physicochemical properties of oils, surfactants and water–miscible solvent or mixture of solvents on the size distribution of nanoemulsions [14]. As well as being drug delivery vehicles in their own right, the formation of a nanoemulsions is the first step in the preparation of nanocapsules and nanospheres by nanoprecipitation [15 ? bonne référence] and in interfacial polycondensation combined with spontaneous emulsification [14, 16]. These two techniques require a spontaneous emulsification step under similar optimised conditions [16, 17].

Droplet size distribution is one of the most important physical properties of a nanoemulsion and depends on the spontaneity of emulsification. Emulsification spontaneity is a poorly defined term which has to take into account not only on the speed of the emulsification process, but also the volume and particle size distribution of the final emulsion [14, 18].

The sense of a simple emulsion (water-in-oil or oil-in-water, commonly abbreviated as w/o or o/w) is defined mainly by the volume ratio of the two liquids, their order of addition and the nature of the emulsifier [19]. In special cases in which spontaneous emulsification can occur, the spontaneity of the emulsification process depends mainly on the following variables: interfacial tension, interfacial and bulk viscosity, phase transition region and surfactant structure and concentration [20, 21]. For other methods of emulsification, an energy input is required, particularly when a small droplet size is required [22]. This energy can be supplied by mechanical means (stirrer, colloid mill, mixer, valve homogenizer) or as

ultrasound [21, 23, 24]. The aim of this study was to obtain a PenGB nanoemulsion formulation, which is suitable for intravenous administration and stable during long term storage. To this end, different protocols for emulsification were tested.

Materials and Methods

Materials

Epikuron 200 was obtained by LUCAS MEYER, Poloxamer 188 was supplied by BASF, Isopropyl Myristate (IPM) was obtained by Sigma, Mygliol 812 was supplied by CONDEA, Phosphate buffer solution (pH=7), Deionized ultrapure distilled water was obtained with a Milli-Q Plus equipment, Methanol and Benzathine penicillin G were obtained by Sigma Sigma–Aldrich (Sao Paulo, Brazil).

Methods

Emulsion preparation: Formulations with the same composition were emulsified by different processes: mechanical agitation (UT), ultrasound (US) and spontaneous emulsification (SE). US nanoemulsions were prepared using an ultrasonic device (marquee, modèke) for 1 minute at amplitude of 30%, while UT systems were produced using an Ultraturrax at 13 500 rpm for 3 minutes. SE nanoemulsions were obtained by the spontaneous emulsification process. The drug is mixed with the oil (Mygliol or IPM). The lipophilic emulsifier (Eykuron 200) is dissolved in a solution of metanol and added to the oil and drug dispersion, resulting in the oily phase. The hydrophilic emulsifier (poloxamer 188) is dissolved in water forming the aqueous phase. The oily phase is then slowly added into the aqueous phase under moderate magnetic stirring, forming the nanoemulsion. Solvents and most of the water are removed under reduced pressure resulting in a formulation concentration of 10% (v/v) of the initial volume from the aqueous phase.

After optimization of the formulation (Table 1) 1mg/mL of PenGB was added to selected systems.

Table 1. Composition of the nanoemulsions samples studied

Sample	Epikuron	IPM	Mygliol	Buffer	Water	Poloxamer 188
TFA-IPM 1	125	250	-	25	-	125
TFA-IPM A	125	250	-	-	25	125
TFA-IPM B	125	250	-	25	-	250
TFA-IPM AB	125	250	-	-	25	250
TFA-Mygliol 1	125	-	250	25	-	125
TFA-Mygliol A	**125**	**-**	**250**	**-**	**25**	**125**
TFA-Mygliol B	125	-	250	25	-	250
TFA-Mygliol AB	**125**	**-**	**250**	**-**	**25**	**250**
TFO 1	**125**	**-**	**250**	**-**	**25**	**125**
TFO A	125	250	-	-	25	125
TFO B	**250**	**-**	**250**	**-**	**25**	**125**
TFO AB	250	250	-	-	25	125

Particle size: The average particle size and size distribution of the nanoemulsions were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments). The measurement was carried out at 25ºC and at a fixed angle of 173° with the samples diluted approximately 10 times with purified water. The particle size of the nanoemulsions was described by the cumulate mean (z-average) diameter and the size distribution was described by the polydispersity index (PdI) and the size distribution graph. Measurements were performed three times for each point. The analyses were performed at Day 1, 5, 10, 15 and 30.

Stability Test: Samples of 6 mL of each emulsion were observed in the Turbiscan MA 2000 (Formulaction – France) in the back-scattering mode at 35°C for 25 min. Measurements were performed three times for each point. Analyses were performed at intervals for 30 days.

Physical-chemical characterization: the conductance of nanoemulsions was determined at room temperature using a CDM230 Conductivity Meter (MeterLab - France). The cell was calibrated by 0.1M KCl. A PHM220 pH Meter (MeterLab - France) was used for the determination of the pH value of the samples at room temperature. The measurements were performed at Day 1, 5, 10, 15 and 30.

Rheological behaviour: The rheological properties were measured using a rotational rheometer Rheostress 600 (HAAKE) at 25ºC. The rheometer was equipped with a stainless steel cone/plate measurement device of 35 mm in diameter, a cone angle of 2° and a gap of 105 µm. The samples were placed on horizontal plate and the cone was position on top.

Transmission Eletronic Microscopy (TEM): For observation by TEM, a drop of nanoemulsion was placed on cooper electron microscopy grids. Before analysis, the nanoemulsions were stained by a 1% phosphotungstic acid aqueous solution. TEM analysis was performed using a Philips EM208 (1996) instrument equipped with a wide- field CCD camera.

Antimicrobial activity: The antimicrobial activity of PenGB nanoemulsions was compared to that of a suspension of PenGB against *Streptococcus pyogenes* and *Staphylococcus aureus*. The inoculums were previously cultivated in solid Colombia Sangue and TSA medium, respectively, for 24h. The bacterial suspension (what concentration) was prepared in BHI medium and increasing concentrations of PenGB nanoemulsions were added to the samples. The samples were incubated at 37 ºC for 24h with each concentration in triplicate. The presence of turbidity was used to analyze the bacterial growth. As a positive control, 1.0 mL of bacterial suspension was added to 10 mL of medium and evaluated at the same time as the samples.

Results and Discussion

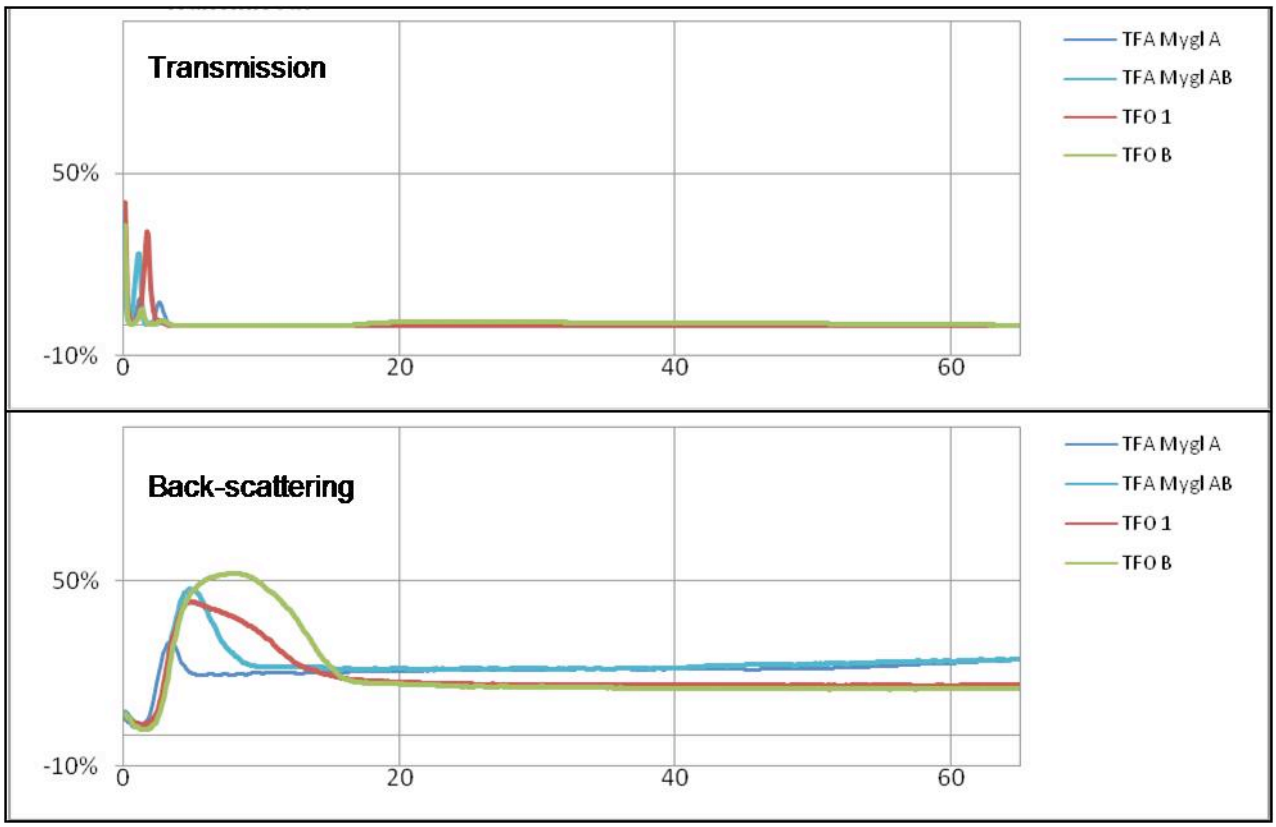
It is important that emulsions to be used as drug delivery vehicles are sufficiently stable. Emulsions may remain practically visually unchanged for several months, but will finally return to their stable state, which is a phase-separated system. In this process, two kinds of phenomena can be discriminated. Firstly, reversible phenomena, including particle aggregation and migration, occur. Secondly, the irreversible processes which are related to particle size modification happen [13, 25-27]. Reversible flocculation of droplets can be followed by creaming or sedimentation, depending on the respective densities of dispersed and continuous phases [18]. The migration rate of the particles of the dispersed phase is governed by Stoke's law [6].

The stability of an emulsion is dependent on the droplet size. The two fundamental processes occurring during emulsification are drop rupture and droplet coalescence. These processes occur concurrently, and the relative rates of the two processes determine the final drop size. Surfactants will affect both these processes (a) by reducing the interfacial tension and interfacial energy, thereby promoting rupture, and (b) by providing a barrier to coalescence via interactions between the adsorbed layers on two colliding drops [26, 28].

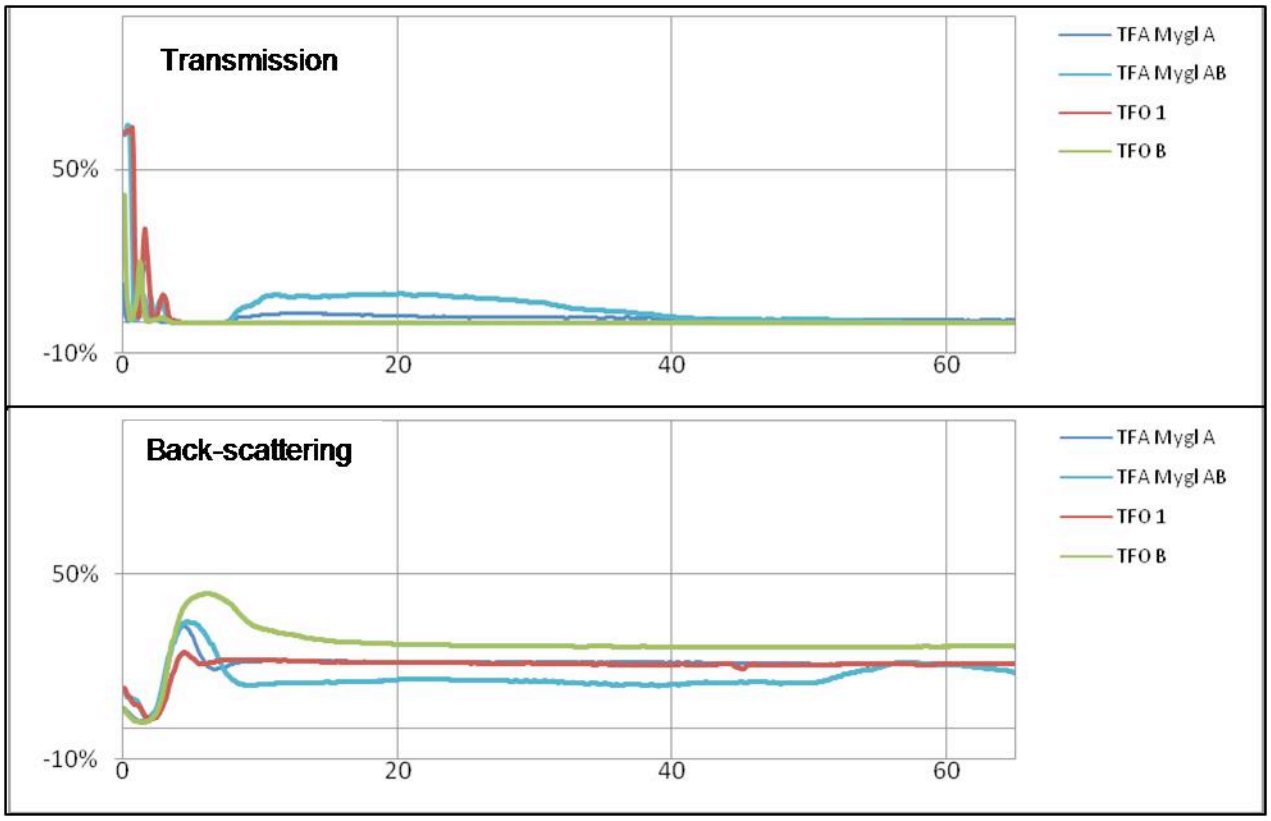
Emulsion stability was studied by visual inspection and also, by Turbiscan MA 2000. This instrument is a tool for characterizing liquid dispersions, which are placed in a glass tube and scanned at all vertical levels by a near-infrared light source. Two types of signal are detected: with a double detection mode: transmission and back-scattering [29]. Figures 1A, 1B and 1C summarize the behavior of nanoemulsions over 30 days. Most of the curves reporting back-scattering intensity data for nanoemulsions at 25°C for 15 min show increasing creaming as a function of time. Since the continuous and dispersed phases are immiscible, Ostwald ripening can be neglected and destabilization occurs only by coalescence. However, there are large differences in stability between the systems prepared by different methods. The most stable systems were produced by spontaneous

emulsification, followed by the US systems. The stability results are correlated with droplet size differences, since the preliminary results regarding the stability of emulsions obtained by three different techniques indicate a much higher stability of US and SE made emulsions. The data indicate that much smaller drops are obtained with gthe US and SE methods compared with UT. Ultrasound was found to produce emulsions with very small particle sizes, which were more stable than those preparedwith the Ultraturrax.

A



B



C

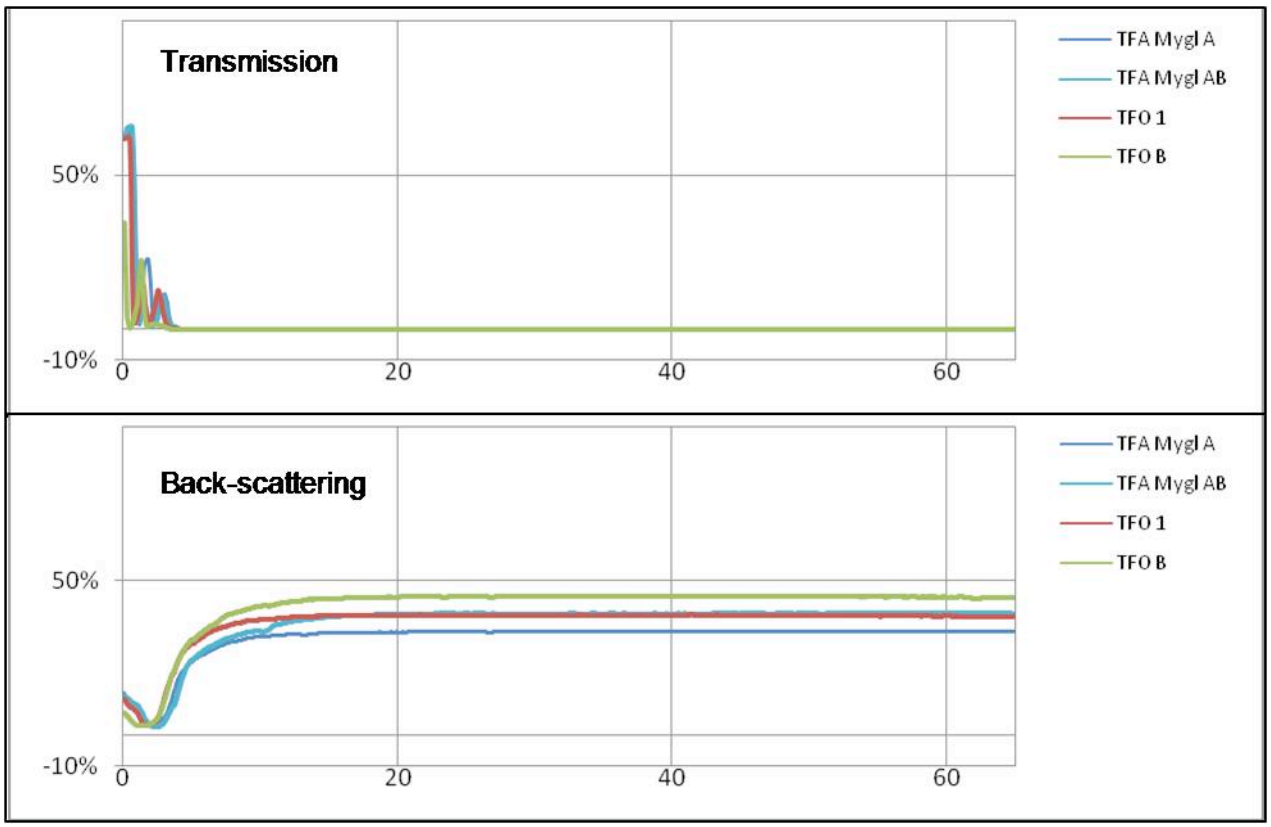


Figure 1. Back-scattering results of the stability nanoemulsions after 30 days. A (ultraturrax sample), B (Ultrasound samples) and C (spontaneous emulsifications samples

Table 2. Results of droplet size and polydispersity index values of PenGB nanoemulsions after 24 h of the preparation; and results of the periode of stability

Method	Sample Name	Record	Z-Ave (d.nm)	Pdl	Stability
US	TFO 1	1	213	0,34	15 days
		2	209	0,37	
		3	210	0,37	
	TFO A	1	262	0,26	15 days
		2	268	0,37	
		3	272	0,46	
EE	TFO 1	1	217	0,07	30 days
		2	207	0,09	
		3	209	0,09	
	TFO A	1	199	0,43	1 day
		2	196	0,47	
		3	193	0,40	

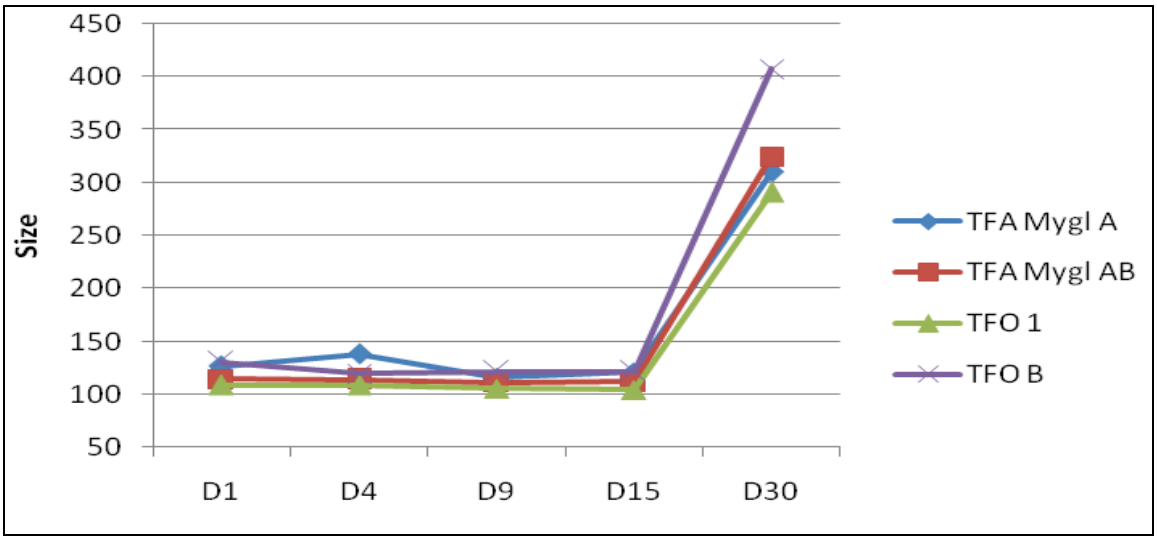


Figure 2. Evolution of the mean droplet size versus time for the samples by spontaneous emulsification prepared.

There was no significant difference between the droplet sizes for US and SE (Table 2). However, when these data are evaluated together with stability analysis, it is clear that SE samples remain stable for longer (Figure 2). Some authors suggest that this might happen

because when low-energy emulsification methods are used, the spontaneous curvature of the surfactant changes during the emulsification process.

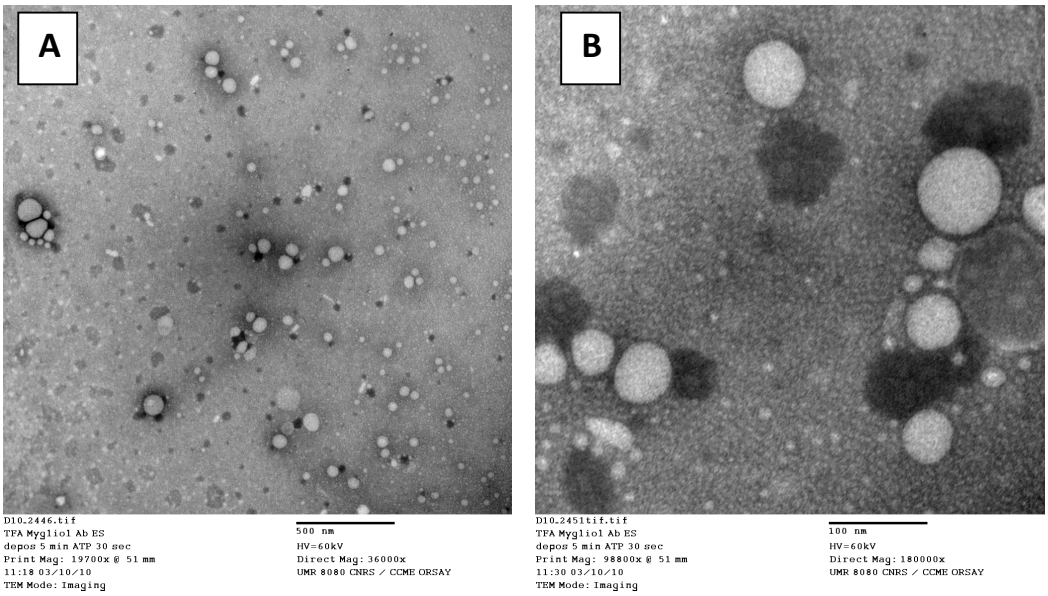


Figure 3 . TEM Photomicrography of TFA Mygliol AB samples by spontaneous emulsification method

The pH (6.5 and 7.5) and conductivity (5-50 µS/cm) results confirm the O/W type and the biocompatibility of the systems prepared. TEM was used to evaluate the morphology of the droplets and the Figures 3A and 3B confirm the previous results and the results obtained by DLS. The rheology results revealed that the samples presented a non-Newtonian behavior at low tension (2 Pa). For higher tension they showed constant viscosity (0.0025 PaS), resulting in a Newtonian behavior. Similar results were found for the samples containing PenGB (results not shown).

The nanoemulsions compositions highlighted in Table 1 were prepared with PenGB. A rise of temperature was observed in the nanoemulsions prepared with the ultrasound method, which could have a deleterious effect on the antibiotic. PenGB was totally encapsulated when SE was used, while it was not well encapsulated in nanoemulsions prepared by US and UT. This could be seen through the observation of some precipitated drug after incorporation.

The *in vitro* activity was determined against *Streptococcus aureus* and *Staphilococcus pyogenes*. Nanoemulsions with or without PenGB were tested in a range of concentrations 30 to 300 ng/mL PenGB or equivalent amount of empty nanoemulsion. Since the MIC of PenGB is 20 ng/mL, bacterial growth was expected only in samples containing lower concentrations. However, when US and UT nanoemulsions were used, all the samples allowed bacterial growth and only samples prepared by SE and containing Miglyol as the oily phase were able to inhibit bacterial growth. This confirms that SE nanoemulsions were the most efficient at encapsulating PenGB.

CONCLUSIONS

The results highlight two important parameters in formulation development: the preparation method and the dispersed phase of the emulsion. SE was chosen as the most suitable method for the production of PenGB nanoemulsions, and Miglyol was found to be the most suitable oil phase. However, further experiments concerning the dispersed phase and the surfactant ratio, as well as the use of a co-surfactant such as egg lecithin is underway. Delivery assays and new activity assays also are in development.

Due tothe difficulty in nanoemulsion development and also because PenGB has little or no solubility in either water or oil, resulting in its final location being usually at the interface of the emulsion droplets; emulsion preparation and scale-up may be problematic. Several strategies could be devised for successful preparation of nanoemulsions containing PenGB. Probably the most promising approach would be the use of co-solvents in which PenGB is highly soluble or the use of micellar solutions to increase the solubility, to produce a PenGB that could be added extemporaneously to a nanoemulsion or a self-nanoemulsifying systems (SNEEDS) to yield a *de novo* preparation.

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DISCUSSION

Cette thèse était basée sur l'objectif de développer un système de délivrance pour la PenGB qui pourrait être utilisé pour le traitement prophylactique du rhumatisme articulaire aigu. Cette maladie, carrément négligenciée, represente une important question de santé publique pour le Brésil et la plupart des pays en développement. Le traitement de référence adopté jusqu'à ce jour est basé sur des injections mensuelles d'une suspension de PenGB. Les échecs bactériologiques de la pénicillinothérapie ont été constatés par divers auteurs (1-5), et la connaissance de tous les inconvénients du traitement comme nous avons montré dans le premier chapitre de cette thèse justifie l'importance de la recherche de nouvelles alternatives de traitement pour cette maladie.

En 2004, ce projet a reçu un financement initial du *Banco do Nordeste* pour sa mise en œuvre. Cette banque crée des outils de développement dans le nord-est du Brésil, qui est reconnu comme région la plus pauvre du pays. Grâce à ce financement, nous avons pu équiper le laboratoire de systèmes dispersés (LASID – UFRN) avec les outils basiques pour le début du développement de la recherche scientifique en médecine et en nanosystèmes.

Pour réaliser ce projet, le travail expérimental a été divisé en 3 parties:

1. **Des études de pré-formulation.** En dépit d'être une molécule assez connu dans la clinique, la PenGB présente des caractéristiques défavorables au développement des nanosystèmes.

2. **Adaptation d'une méthode de dosage spectrophotométrique pour les substances en quantités significativement faible**. En raison des contraintes dans les infrastructures que nous avons au LASID, nous sommes souvent amenés à adapter les protocoles et des expériences. Dans ce contexte, le développement et la validation d'une méthode spectrophotométrique pour PenGB qui a été similaire dans le plan technique et le plus approprié au niveau de la vitesse et de coût par rapport au HPLC, a été nécessaire dans le première année de ce travail.

3. **Développement de nanosystèmes émulsionnée contenant PenGB**. Considérant que PenGB est une molécule peu soluble dans l'eau, l'incorporation de cette molécule dans la phase dispersée d'un système d'émulsion semble une alternative très acceptable, tant du point de vue technologique que du point de vue clinique.

1. Essais de pré-formulation :

Les tests effectués sur les premiers travaux expérimentaux de cette thèse a permis d'évaluer les paramètres physico-chimiques de la PenGB en termes de : sa solubilité, sa stabilité et sa lipophilie. Ces tests sont importants pour envisager des stratégies pour accroître la solubilité apparente de cette molécule, et pour comprendre les problèmes éventuels qui peuvent survenir lors de l'élaboration de la formulation et, peut affecter les caractéristiques de la formulation finale. Les caractéristiques de solubilité de la PenGB ont été étudiés par rapport aux effets de divers facteurs comme le pH, l'effet ionique, la température.

A partir des ces informations, une stratégie pour améliorer la solubilité en utilisant des solutions micellaires de désoxycholate de sodium a été réalisée. L'influence de la force ionique dans ces systèmes a été analysée et nous avons pu comprendre que l'incorporation de la PenGB dans des solutions micellaires peut être une alternative pour accroître la solubilité en solution aqueuse, ainsi qu'une approche prometteuse pour moduler les caractéristiques de cette molècule avant d'être incorporés dans les systèmes lipides.

2. Adaptation d'une méthode de dosage spectrophotométrique

Les méthodes officielles d'analyse utilisées pour quantifier des médicaments sont basées sur l'utilisation de méthodes gravimétriques, spectroscopiques dans l'ultraviolet/visible (UV-Vis) ou chromatographiques [6-10]. Toutes ces méthodes ont leurs avantages et inconvénients selon leur zone d'application. L'utilisation de la méthode traditionnelle UV-vis spectrophotométrique dans l'analyse pharmaceutique et biomédicale a déjà été démontré dans plusieurs études [11-20]. Ce fait indique que les méthodes spectrophotométriques sont un possible choix pour des analyses quotidien dans les laboratoires de recherche et de controle de qualité des médicaments.

Une approche récente pour réduire les variations a été de préparer une courbe d'étalonnage en utilisant la méthode avec une cuvette stationnaire [21]. Cette procédure consiste en une méthode d'interpolation pour obtenir des courbes d'etalonage pour des medicaments et des substances phytochimiques. Le but de cette étude était de développer un simple, rapide, précise et peu coûteuse procédure spectrophotométrique pour la détermination de medicaments dans des préparations pharmaceutiques. Une courbe d'étalonnage a été établie, et de l'exactitude et la précision des résultats ont été déterminés par rapport à une méthode traditionnelle de présentation des résultats d'absorbance.

Cette étude a montré que le remplacement de la méthode traditionnelle pour la méthode avec cuvette stationnaire peuvent être facilement mises en œuvre sans la nécessité d'un changement de solvants et /ou techniques. Dans le tableau 1, les résultats concernes à linéarité de la méthode cuvette stationnaire. Ils montrent donc la capacité d'analyser des échantillons à faibles concentrations, ce qui suggère son avantage potentiel à l'analyse des nanosystèmes, et dans les cas des échantillons où l'extraction est une étape limitant comme en médecine légale et l'analyse toxicologique.

D'autres avantages de cette méthode comprennent son faible coût et leur faible besoins de solvant. Ces deux propriétés montrent des avantages réels de la méthodologie de la cuvette stationnaire pour l'analyse des produits pharmaceutiques et de composés phytochimiques par spectrophotométrie UV-vis. En fait, en utilisant cette méthodologie, la consomation de réactifs a été réduit de cinq fois par rapport à celle traditionnelle. En ce qui concerne le temps d'analyse, tout en comparant avec la méthode traditionnelle, la procédure cuvette stationnaire utilise seulement 30% du temps. Un autre point important est la réduction de la quantité de matériel de laboratoire (tels que les fioles jaugées, béchers, pipettes etc), ce qui réduit les erreurs aléatoires lors de l'élaboration de la procédure. Tous ces aspects contribuent à la réduction des coûts de cette nouvelle méthode.

Les résultats présentés (tableau 2) confirment qu'il n'y a pas de différence significative entre la méthode traditionnelle d'analyse de produits pharmaceutiques par spectrophotométrie UV-VIS et la méthode de la cuvette stationnaire et qu'il peut être utilisé pour différentes molécules avec une précision significative (Tableau 3), confirmant les données provenant de travaux antérieurs.

La méthode décrite dans ce travail offre une nouvelle possibilité dans le dosage des préparations galéniques. Ses caractéristiques intéressantes, telles que: la préparation d'échantillon minimale, la vitesse d'analyse, et la simplicité de fonctionnement, ainsi que la capacité inhérente d'analyser toutes sortes de matériaux indépendamment de leur forme physique et leur état d'agrégation, d'ouvrir de larges possibilités d'utilisation de cette technique dans l'industrie, l'environnement et les sciences biologiques. Les autres avantages par rapport à la méthode actuelle comprennent la limite de détection basse ainsi qu'une grande précision qui influent sur la sensibilité et la fiabilité de la méthode.

	Amoxicillin		***5*-amino salicylic acid**		**Mandelic Acid**		**Hydroclorothiazide**	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
***C* (mM)**	$0.20x10^{-3}$ – $0.90x10^{-3}$	$0.67x10^{-5}$ – $0.85x10^{-4}$	$0.80x10^{-5}$ – $0.26x10^{-3}$	$0.40x10^{-5}$ – $0.30x10^{-4}$	$0.60x10^{-3}$ – $0.40x10^{-2}$	$0.33x10^{-4}$ – $0.30x10^{-3}$	$0.17x10^{-4}$ – $0.50x10^{-4}$	$0.42x10^{-6}$ – $0.40x10^{-5}$
n	10	12	10	12	10	12	10	12
b	0.0324	0.0085	0.023	0.0685	-0.0077	-0.0187	0.0059	0.0127
a	0.0025	0.0255	0.0119	0.1075	0.0014	0.0187	0.0517	0.7089
R^2	0.9955	0.9999	0.9934	0.9984	0.9994	0.9999	0.9988	0.9999

Tableau 1. Résultats de l'étude de linéarité pour la méthode classique (1) et la méthode de la cuve stationnaire (2). C= gamme de concentration pour une réponse linéaire ; n = nombre d'échantillons analysées, b = pente, a = intercepte, R^2 = coefficient de corrélation

One-way analysis of variance	Amoxicillin		*5*-amino salicylic acid		Hydroclorothiazide		Mandelic Acid		Amphotericin B	
	1	2	1	2	1	2	1	2	1	2
P value	0,9979	0,9943	0,9474	0,9646	0,0636	0,9810	0,9420	0,4937	0,9758	0,9791
P value summary	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns
Are means signif. different? (P < 0.05)	No	No	No	No	No	No	No	No	No	No
Number of groups	9	9	9	9	9	9	9	9	9	9
F	0,1269	0,1702	0,3411	0,3003	1,940	0,2442	0,3527	0,9324	0,2643	0,2521
R square	0,01238	0,01357	0,03110	0,02012	0,1485	0,02383	0,03366	0,07280	0,02295	0,02216

Tableau 2. Les résultats de l'étude de la robustesse avec la méthode classique (1) et avec la méthode de la cuvette fixe (2).

	Sample 1	Sample 2	Sample 3	Mean	SD	%CV
Analyst 1	0,6150	0,6090	0,6090	0,611	0,0034	0,5669
Analyst 2	0,6850	0,6890	0,6840	0,686	0,0026	0,3856
Analyst 3	0,5380	0,5570	0,5540	0,549	0,0102	1,8582

Tableau 3. Précision intermediaire pour la méthode de la cuvette stationnaire avec Amoxicilline. SD = écart type, %CV = coefficient de variation

3. Développement de nanosystèmes contenant émulsionnée PenGB

Contrairement aux émulsions classiques, les émulsions qui ont des gouttelettes de taille nanométrique, tels que les microémulsions et les nanoémulsions ont été choisis comme le système de l'intérêt pour incorporer PenGB. Microémulsions, un exemple de transporteurs colloïdale, sont formé spontanément dans une seule phase d'huile-dans-eau ou eau-dans-huile, stabilisées par un film interfacial de tensioactifs et de co-tensioactifs. Ces dispersions auto-assemblés ont une faible viscosité, très faible tension interfaciale, bonne stabilité, une grande capacité de solubilisation, l'homogénéité macroscopique, et l'hétérogénéité microscopique (microdomaines) (22-27), en raison de tous ces avantages de ces systèmes par rapport aux autres systèmes pour la libération soutenue, comme a été discutés dans la partie des travaux antérieurs de cette thèse. Le développement d'un système d'émulsion comporte plusieurs étapes critiques, depuis le choix des composants, jusqu'au développement de la formulation elle-même (28-32). Quand le système est développé pour une administration parentérale, le choix des composants doit passer par une sélection rigoureuse d'huiles et de tensioactifs qui sont acceptées par les organismes de réglementation comme approprié pour cette voie d'administration en termes de sécurité et une faible toxicité. Toutefois, ce facteur a considérablement limité la gamme de produits qui pourraient être utilisés. Nous avons commencé notre étude avec des huiles végétales composée de triglycérides à chaîne longue comme le huile de tournesol et le huile de sésame, qui sont utilisés de façon courante par voie intraveineuse pour des objectifs nutritionnels, en plus, nous utilisons également dans nos études preliminaires des huiles composés des triglycérides à chaîne moyenne comme celles provenant de l'huile de ricin, qui sont déjà inscrites dans la littérature scientifique pour une utilisation dans les systèmes ayant des objectifs similaires.

Malgré une grande variété d'agents de surface, seul un nombre restreint peut être utilisé pour les préparations parentérales. Le surfactant le plus courant pour de telles émulsions est de la lécithine. Dans ce travail, la lécithine et d'autres agents de surface tels que le Cremophor EL, le Tween 20 et le Span 80 ont été utilisés. Le choix des agents de surface est fondé sur les résultats publiés d'études scientifiques. Bien que nous savons que il faut nécessairement ajouter des autres ingrédients dans la formulation, comme antioxydants et agents osmotiques, à ce phase de développement de la formulation n'a pas été accordé une attention à ces éléments.

Avant de commencer le développement réel de la formulation, les essais sur le HLB des huiles usagées se sont avérés nécessaires. En ce qui concerne l'Mygliol 812, son HLB a été bien établi par Macedo et al (33) dans notre groupe de recherche. Des études similaires

et, pas encore publiées, ont été développées pour la détermination du HLB d'huiles végétales (de tournesol et de sésame) utilisées dans ce travail.

Il est connu que d'un mélange ordinaire de l'eau, d'huile et de surfactant sont capables de former de nombreux types de structure en fonction de la concentration de chaque composant (34-37). Selon ces informations, la première étape dans le développement d'un système de délivrance de médicaments en systemes emulsionéé est la construction de diagramme des phases soit ternaire ou pseudoternaire, avec le but d'étudier le comportement des phases qui seront formé à partir de mélange des composants choisi. Le diagramme de phase est essentiel pour comprendre le rôle de chaque composant dans le système formé. Ils sont également tenus d'étudier la capacité de solubilisation des formulations obtenues.

Après la construction des différents diagrammes pseudoternaires, nous avons pu choisir les meilleurs composants qui seront utilisés dans le développement de systèmes. Nous avons construit plusieurs diagrammes, répartis essentiellement en deux groupes: le premier groupe contenat des diagrammes avec le Mygliol comme la phase lipophile, avec combinaisons d'agents de surface. Dans le deuxième groupe, le système tensioactif a été fixé à un mélange de Tween 20 et Span 80 (1:1) et différents phases lipophiliques ont été utilisées. Même si les alcools à chaîne courte peuvent avoir un rôle en tant que co-agent de surface à la stabilisation du système et par conséquent augmenter la superficie de la region de microémulsion, dans cette étude il a été décidé de ne pas pas utiliser d'alcools dans la formulation, en raison de nombreux inconvénients que ces adjuvants peuvent provoquer. Dans chaque diagramme construit, des échantillons dans la région de microémulsion ont été choisis à analyser et caractériser. Les formulations présentant des meilleures caractéristiques de stabilité ont été choisies pour poursuivre l'étude et ont été analysés en termes de caractérisation physique et chimique. Il a été observé à partir de la caractérisation par microscopie optique polarisée qu'il y a une variation, en fonction de la composition, entre les structures bicontinuum ou organisée sous forme de gouttelettes. Il est également été observé que bien que certains systèmes ayant des caractéristiques de l'isotropie, la transparence et la stabilité, certains ne peuvent pas résister à dilution simple. Dans ce cas, le concept de microémulsion avec le sens des systèmes thermodynamiquement stable devrait être révisé, et d'autres façons de nommer ces systèmes devraient être considérées.

Outre une importante question technologique, du fait que ces systèmes ne résistent pas à la dilution génère une question théorique. Ses caractéristiques d'isotropie, la transparence et la stabilité sont exactement conformes à celles proposées dans la littérature pour des systèmes appelés microémulsions. Toutefois, dans le test de dilution, la formation

spontanée des nanoémulsions vraies a attiré notre attention. Selon certains auteurs des systèmes peuvent être dénommés microémulsions s'ils restent stable après l'étape de dilution, en cas de conversion en nanoémulsions, ces mélanges d'huile, d'eau et de agent de surface, doit *à priori* être appelé systèmes « auto nanoémulsifiants ».

Le taux d'incorporation de la PenGB dans les microémulsions développé dans ce travail s'élève à environ 10% et la stabilité macroscopique de ces systèmes a atteint plus de 12 mois. Sur cette base, nous proposons ces mélanges en tant que solvant pour PenGB, la nanoémulsion à partir de laquelle se forment lorsque extemporanément préparée juste avant l'application. Pendant le développement de tests avec des microémulsions, on s'aperçut que les nanoémulsions pourraient avoir de potentiels avantages à incorporer la PenGB. Sur cette base, le développement des nanoémulsions ont été effectués. Plusieurs modèles expérimentaux ont été réalisés dans lequel les variables ont été analysées comme formulation: phase lipophile (Mygliol et Isopropylmyristrate), phase hydrophile (l'eau et le tampon phosphate) et le rapport entre les tensoactifs. En outre, nous avons analysé trois techniques de production qui différent par la méthode d'agitation: homogénéisation-dispersion (Ultraturrax), émulsification par ultrasons et émulsification spontanée.

Les échantillons ont été produits et caractérisés d'abord sans PenGB, et les plus stables ont été choisis pour l'incorporation de l'antibiotique. Les résultats ont montré que le PenGB n'a eu pas d'influence sur les caracteristiques physico-chimiques des systèmes. Les nanoémulsions avec PenGB produits par ultraturrax et par ultrasons n'ont pas une activité *in vitro*, contrairement à celles produites par émulsification spontanée. Ce fait nous amène à penser que la force d'agitation par ultraturrax ou par ultrasons n'est pas suffisante pour intégrer le médicament dans les systèmes. Les analyses par microscopie optique ont montré la présence d'un précipité dans les formulations produit par ces techniques. Compte tenu des propriétés physico-chimiques de la PenGB étudiées dans la première partie de ce travail, nous pouvons conclure que l'utilisation de co-solvabilité est essentielle à l'intégration réussie de cette molécule dans les systèmes nanoémulsionnés. Dans ce cas, en dépit de sa toxicité, le meilleur solvant organique au cours du processus d'émulsification spontanée serait le méthanol, car il est capable de solubiliser à la fois les composants lipophiles et la PenGB, garantissant ainsi que le médicament sous forme moléculaire au cours de la formation de nanoémulsion.

Bien que les expériences relatives à l'incorporation de PenGB dans les systèmes microémulsionées ne soient pas définitives, les discussions et les informations de ces résultats sont décisives pour la poursuite de ce projet. Les résultats obtenus qui suggèrent que la PenGB doit être dans sa forme moléculaire pour être intégrée à nanoémulsion

confirment les informations théoriques sur les façons d'augmenter la solubilité apparente de médicaments insolubles et dirigent les prochaines étapes de l'élaboration de systèmes de liberation prolongée pour la PenGB, tel que nanoémulsions ou des systèmes paticulaire dérivéesde celas.

Dans ce travail la technique d'augmenter la solubilité utilisée a été l'utilisation du méthanol comme co-solvant, toutefois, les résultats obtenues dans la première partie de ce travail concernant les solutions micellaires de PenGB, suggèrent la micellisation pourrait être un autre soutien technologique à l'incorporation de PenGB. Des expériences de solubilisation de la PenGB avec des solutions micellaires de Pluronic ont également étés développées. Notre perspective est d'utiliser ces solutions micellaires comme la phase aqueuse dans la production de microémulsions et nanoémulsions. Bien que ces études ne soient pas encore finalisées, elles donnent des résultats prometteurs. De plus, des essais de production de nanoparticules en utilisant ces nanoémulsions comme première étape sont également en cours.

L'ensemble des résultats obtenus dans cette thèse s'intègre dans l'une des lignes de recherche du LASID Ce laboratoire travaille sur le développement de base et la caractérisation des nanosystèmes émulsionnée, mais les résultats de ce travail apportant un soutien à d'autres lignes de recherche en cours, tels que le développement des émulsions pour l'érythème fessier, émulsions cosmétiques à base d'huile de tournesol et de microémulsions d'huile de sésame, pour les vehiculer d'autres médicaments comme le kétoconazole et l'amphotéricine B. Les résultats de ces collaborations ont été présentés lors de conférences tout au long de la thèse et sont présentés dans les annexes.

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CONCLUSION GENERALE

Malgré les récentes innovations en technologie pharmaceutique dans la conception et mise au point des systèmes galéniques qui permettent de moduler la distribution des certains medicaments, il reste des molécules thérapeutiques clés qui sont toujours administrées dans de systemes traditionnels de libération. Ceci a des conséquences pour leur activité pharmaceutique, dont les limitations seront plutôt dues à l'inadéquation de la forme galénique qu'aux propriétés de la molécule elle-même.

Le but de notre travail a été d'associer un antibiotique avec des propriétés physicochimiques défavorables (la benzathine pénicilline G - PenGB) à des systèmes colloïdaux émulsionnés tels que les nanoémulsions et microémulsions.

La première partie experimentale a été réalisée au Brèsil, pendant laquelle nous avons mise au point des essais de prè-formulation. Ces essais ont fourni des informations importants sur les propriétés physico-chimiques, la solubilité et la stabilité de la PenGB qui ont permis de mettre au point de solutions micellaires. La méthode pour l'incorporation de la PenGB dans des solutions micellaires a été montrée être simple et rapide, et il a également été capable d'augmenter sensiblement la quantité des molècules incorporés par rapport au solutions aqueuse. Par conséquent, les solutions micellaires semblent être une approche prometteuse pour améliorer la solubilité apparente de la PenGB soit pour son usage thérapeutique soit comme une stratégie visant à accroître son chargement en systèmes emulsionées.

Dans la premiere partie, nous avons développé et validé une méthode analytique quantitative par spectrophotométrie UV-visible qui s'est montrée plus performante que la méthode proposée dans la littérature. Ce protocole expérimental a également permis de faire une étude de pré-validation et d'évaluer les caractéristiques de la méthode développée par l'analyse des paramètres de la linéarité, la portée, précision, l'exactitude et les limites de quantification et de détermination. Malgré l'existence d'une méthode d'étalonnage déjà établie, certains inconvénients à ce mèthode fait ressortir la nécessité pour une nouvelle méthode comme celle qui a été proposée dans notre travail, avec des caractéristiques de performances similaires, ou même meilleures, et des avantages dans des domaines tels que la facilité, la rapidité et le faible coût. Les facteurs qui rendent la méthode de la Cuve Stationnaire superieur à la mèthode traditionnelle sont d'une grande importance, puisque l'intention de cette étude était de proposer une méthode pour l'analyse de routine aux laboratoires des l'enseignement et de recherche. La méthode de la Cuve Stationnaire est utilisée actuellement dans notre groupe pour l'analyse de la PenGB et a également été employée pour l'analyse des différentes molécules ainsi que les extraits vegetaux.

La deuxième partie experimentale a été realisé en France. Cette étude a été dediée à la mise au point des nanosystemes émulsionés pour l'encapsulation de la PenGB. Plusieurs diagrammes pseudoternaires ont été construits utilisant différents composants dans le but d'optimiser les formulations de microémulsions pour la voie parenterale. Cependant, différentes techniques de caractérisation ont montré que bien que le système développé possède la plupart des caractéristiques nécessaires pour être considéré comme une microémulsions, après l'essai de dilution leur structure a changé de celle d'une microémulsion à celle d'une nanoémulsion. En ce qui concerne l'incorporation de PenGB, les résultats ne sont pas concluants. Il paraît que la PenGB peut être encapsulée dans les systèmes qui sont stables soit sous forme de microèmulsions (jusqu'à un an) soit sous forme de nanoémulsions après l'étape de dilution (jusqu'à sept jours). Cependant, des expériences supplémentaires doivent être effectuées afin de confirmer ces résultats. Au cours de la deuxième partie, des nanoémulsions chargées en PenGB ont été développées. Plusieurs plans factoriels ont été réalisés afin de choisir la meilleure formulation et la technique de production les plus adaptée. Les formulations ont été caractérisées pour leur stabilité physico-chimique. Les essais d'activité in vitro vis-à-vis deStreptococcus pyogenes et Staphylococcus aureus ont été réalisées sur les échantillons qui ont montré les meilleures caractéristiques galéniques. Les résultats nous à conduit à choisir le Mygliol 812 comme phase lipidique pour la nanoémulsion contenant la PenGB, et la technique d'émulsification spontanée comme protocole de préparation.

Au terme de ce travail, un certain nombre de points restent à éclaircir. Sur le plan galenique, une étude plus approfondie de la microorganisation des microèmulsions devrait être realisée afin de comprendre pourquoi ces systèmes changent de structurs après la dilution. Cette étude pourrait également expliquer les interactions entre les composants des systemes et la molécule de PenGB. Parmi les techniques physico-chimiques qui pourrait être utilisées, la RMN et le DSC devrait être d'un apport considerable.

Sur le plan biologique, des études de libération in vitro et in vivo de PenGB des nanoémulsions chargées devrait être réalisée, afin de prévoir l'efficacité thérapeutique. Enfin, dans l'avenir, il serait indispensable de continuer les études de développement galéniques qui permettront arriver à la formulation finale d'un système de libération prolongée chargée en PenGB. Ce système pourrait être soit un système lipidique émulsioné, soit un système particulaire qui pourrait être développé à partir des microèmulsions et nanoemulsions mises au point pendant ce travail.

En conclusion, l'ensemble des études que nous avons menées et les résultats obtenus peuvent être considérée comme la partie debutant dans le cadre du développement

d'un nouveau système galénique pour la liberation de PenGB destiné au traitement de la fièvre rhumatoïde.

ANEXES

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