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# Preparation and Evaluation of W/O/W Multiple Emulsion Containing Naltrexone Hydrochloride: A Pilot Study

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***SUMMARY*. W/O/W multiple emulsions containing naltrexone (NTX) hydrochloride were prepared by a two-step emulsification method at 20 ºC. Characterization of the developed system was evaluated and the release kinetics of the drug was determined. The tissue response to the injection of the multiple emulsion in mice was observed by histological analysis. The entrapment efficiency of NTX hydrochloride in W/O/W multiple emulsion was 97.72 ± 0.8 % and the mean diameter of the multiple globules was 18.6 ± 7.7 *μ*m. The main *in vitro* drug release mechanism observed for the developed system is supposed to be a swelling-breakdown phenomenon after dilution of the emulsions under hypo-osmotic conditions. Biocompatibility studies showed that the multiple emulsion was well tolerated as no significant toxic reaction was observed. The W/O/W multiple emulsion containing NTX hydrochloride may represent a potential alternative dosage form for the treatment of alcohol dependence.**

## INTRODUCTION

According to Yin *et al.* [1] and Costantini *et al.* [2], a glance at literature on alcohol dependence will reveal a catalog of medical complications and the main approaches to the treatment of alcoholism include brief intervention, behavioral and cognitive-behavioral approaches, psychosocial and motivation-enhancement methods, and pharmacotherapies. It is ascertained that alcohol acts on the opioid receptor sites, so an opioid antagonist could be effective in the treatment. As pharmacotherapies, only three medications are officially available for the treatment of alcoholism: disulfiram (Antabuse™), naltrexone (Revia™) and calcium cetylhomotaurinate (Acamprosate™).

Naltrexone (NTX), an opioid antagonist, blocks intrinsic properties of psychoactive substances that act on the μ, κ, and σ opioid receptor sites by competitive occupation. By blocking these sites, NTX prevents the reinforcing effects of alcohol consumption [3]. However, oral naltrexone is associated with a number of limitations which may subsequently reduce its efficacy in treating alcohol dependency and could increase the risk of relapse: oral naltrexone is readily absorbed through the gut, it suffers significant first pass hepatic metabolism with an oral bioavailability of only 5-40% and daily dosing results in fluctuating plasma concentrations of the drug during the day. To overcome these limitations, attempts have been made to develop an injectable extended-release formulation of naltrexone, including encapsulation into biodegradable polymer microspheres [4-6], polymeric implants [7,8], buccal tablets [9] and polymeric complexes [10]. Extended-release delivery of drugs has several advantages to optimize the maintenance of treatment: these systems improve compliance without the restrictions of daily medication and would eliminate this first pass metabolism [6].

The multiple emulsions are three-phase dispersion in which oil droplets containing an in-

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ternal aqueous phase are dispersed in an external aqueous phase. These emulsions have significant potential in the pharmaceutical field since they provide prolonged release of the drugs encapsulated [11]. Prolonged drug release systems using w/o/w multiple emulsions entrapping protein [11-13], rifampicin [14,15], tacrolimus [16], isoniazid [17], tegafur [18], vancomycin [19], diclofenac sodium [20] and antiseptics [21] have been studied.

In the present study, we aimed the development of multiple emulsions containing NTX. Characterization of the developed system was evaluated and the release kinetics of the drug was determined. The tissue response to the injection of the multiple emulsion in mice was observed by histological analysis.

## MATERIALS AND METHODS

### *Materials*

NTX hydrochloride ($C_{20}H_{23}NO_4.HCl$) was kindly donated by Cristália Produtos Químicos Farmacêuticos LTDA (São Paulo, Brazil). The oil phase consisted of medium chain triglycerides (Caprylic/Capric Triglyceride - Miglyol™ 812, SASOL Co., Germany) and the polymeric surfactants used were: lipophilic surfactant poloxamer 401 (Pluronic™ L121, BASF Co., Germany) and hydrophilic surfactant polyoxyl-35 castor oil (Cremophor EL™, BASF Co., Germany). These surfactants are used in commercially available injectable formulations [22]. Methanol HPLC grade and diammonium phosphate were purchased from Omega LTDA (Brazil). Ketamine hydrochloride (Ketamin™) was purchased from Cristália Produtos Químicos Farmacêuticos LTDA (São Paulo, Brazil). Ultrafiltrated water was obtained from Milli Q plus, Millipore (USA). All other chemicals were of analytical grade.

### *Preparation of the W/O/W multiple emulsion*

The multiple emulsion was prepared by a two-step process [23]. The primary emulsion consisted of (w/w) 45% oily phase, 5% poloxamer 401 and 50% of an aqueous solution of NTX hydrochloride. The W/O/W multiple emulsion consisted of (w/w) 75% of primary emulsion and 25% of an aqueous solution containing 4% polyoxyl-35 castor oil. The two stages of emulsification were carried out at 20°C using an Eurostar™ stirrer (Ika Works Inc, Brazil). The stirring rate was 3000 rpm for 30 min in the first step and 900 rpm for 20 min in the second. The aqueous NTX hydrochloride solution was prepared by dissolving the drug in phosphate-buffered saline (PBS) at pH 7 to obtain a final NTX hydrochloride concentration of 10.0 mg/ml.

Sodium hydrochloride was added to the external aqueous phase in order to obtain approximately equal osmotic pressure (Micro Osmometer - µOSMETTE, Precision Systems, Rio de Janeiro, Brazil) between the internal and external aqueous phases of the emulsions. The final concentration of the external aqueous phase (NaCl solution) was (1.15% w/v).

### *High-performance liquid chromatography (HPLC) analysis*

NTX hydrochloride analysis was performed by HPLC using a modified version of the method described by Alvarez-Fuentes *et al.* [10]. Briefly, it was determined at 20 °C using a C18 reverse-phase column (250 x 4.6 mm) filled with octadecyl silane chemically bonded to porous silica (5.0 µm, Shim-pack™ LC18, Shimadzu, USA) by a Waters apparatus equipped with an integrator software (Millenium, Waters, USA) and an autosampler model 717plus (Waters, USA). A pump (model 515, Waters, USA) was used at a constant flow rate of 1.0 ml/min. An ultraviolet detector (model 2487, Waters, USA) was used at a wavelength of 288 nm. The mobile phase was a mixture of methanol/purified water/diammonium phosphate 70:30:0.1 v/v/w. The retention time for NTX was 7.04 ± 0.1 min. The calibration curve obtained for NTX hydrochloride from standard solutions containing 200, 100, 50, 25, 12.5 and 6.25 µg/ml of the drug dissolved in the mobile phase was linear (correlation coefficient $r^2 = 0.9991$) and showed the adequate precision of the HPLC method proposed.

### *Characterization of the multiple emulsions developed*

*Macroscopic and microscopic analyses*

Macroscopic analysis was carried out by visual inspection in order to observe the homogeneity of the multiple emulsions after preparation. Microscopic observations were made with an optical microscope (Microscope Olympus BX 41, USA) at 1000 x magnification after diluting in the appropriate external phase. The images were captured using a Sony CCD-IRIS apparatus (Sony, USA). This analysis allowed us to confirm the formation of the multiple emulsion by observing the percentages of simple and multiple globules.

*Determination of the mean diameter of the multiple emulsions globules*

The mean diameter of the multiple emulsions globules were measured by particle counting. For the analysis, the W/O/W multiple emulsions were dispersed in Isoton™ (electrolyte solution), in order to obtain a convenient concentration between 8 and 12%. The mean particle size and the size distribution of the multiple globules were determined using photon correlation spectroscopy (PCS) with a Malvern 4700 photon correlation spectrometer (Malvern Instruments, Malvern, U.K.). The distribution histogram of the particle size for the multiple emulsions was obtained through the graphic representation of the particle diameter according to their frequency [24].

*Determination of the entrapment efficiency*

The entrapment efficiency of NTX hydrochloride in W/O/W multiple emulsion was determined as follows: the W/O/W multiple emulsion was diluted 10-fold in NaC1 solution (1.15% w/v). After centrifugation (3500 x g, 15 min), NTX hydrochloride was extracted from the simple W/O emulsion in the top layer, using the mobile phase of HPLC analysis, in 10-fold excess with respect to the emulsion. After extraction, the aqueous phase was separated by centrifugation (3500 x g, 15 min) and filtered with a Millipore filter (0.45 µm) before HPLC analysis.

*Release kinetics*

NTX hydrochloride release was evaluated by HPLC analysis after dilution (10-fold) of samples (n = 6) of the multiple emulsions developed in hypo (Ultrafiltrated water) and iso-osmotic conditions (NaC1 solution 1.15 % w/v). The flaks containing the diluted multiple emulsion were placed in a water bath BD R02020 (Lauda, Germany) at constant temperature of 37 °C, coupled with a stir plate. The samples were collected periodically at 15, 30, 60, 90, 120, 240, 360, 480 min and 1, 3, 7 and 14 days after beginning the study and replaced by an equal volume of the release medium (Ultrafiltrated water or NaCl solution 1.15% w/v) to maintain sink conditions. The aqueous phase was separated by centrifugation (3500 x g, 15 min) and filtered with a Millipore filter (0.45 µm) before NTX hydrochloride determination. The percentage of NTX hydrochloride released from each formulation was calculated using the following equation: % of NTX hydrochloride released = (Ct/Co) x 100, where Co is the initial concentration of NTX hydrochloride (mg/g) in the multiple emulsions and Ct is the concentration of drug (mg/ml) in the collected samples.

***Tissue response to subcutaneous injection of W/O/W multiple emulsion***

Female outbread Swiss mice, 8 weeks old, weighing 28 ± 2 g, were used to evaluate the biocompatibility of the developed multiple emulsions. The animals were anaesthetized with intra-peritoneum injection of ketamine hydrochloride 40 mg/kg (Cistália, Brazil), shaved over dorsal anterior surface, the site cleaned with a povidone–iodine alcoholic solution and 100 µl of multiple emulsions were subcutaneously injected. The NTX hydrochloride solution alone (100 µl) was also injected in the control animal group. The animals were maintained on standard diet and water *ad libitum*. Within each group, three animals were sacrificed at predetermined intervals at 72 h, 1 and 2 weeks after the injection of the emulsion sample or the NTX hydrochloride solution by ketamine overdose (100 mg/kg). Immediately after animal sacrifice, the surrounding tissues were cut off and fixed in 10% phosphate buffered formalin solution for 48 h. All samples were alcohol-dehydrated, soaked in xylol and embedded in paraffin. Sections were cut using a microtome and stained with hematoxylin and eosin according to standard procedures. Microscopic analysis was performed by the same single blinded examiner, using a light microscope. The study was done following the guidelines for care and use of experimental animals as laid down by the Institutional Animal Ethics Committee.

## RESULTS

***Characterization and in vitro release kinetics***

The W/O/W multiple emulsions containing NTX hydrochloride were easily prepared by the two-stage emulsification procedure. They were macroscopically homogeneous and the microscopic analysis confirmed the formation of the multiple emulsion (Fig. 1). In addition, the entrapment efficiency of NTX hydrochloride in the W/O/W multiple emulsion was correspondent to 97.72 % ± 0.8 and the mean diameter of the multiple globules, measured immediately after emulsion preparation was 18.6 ± 7.7 µm. The analysis of the distribution histogram of the multiple emulsion particle size shows a monomodal form (Fig. 2).

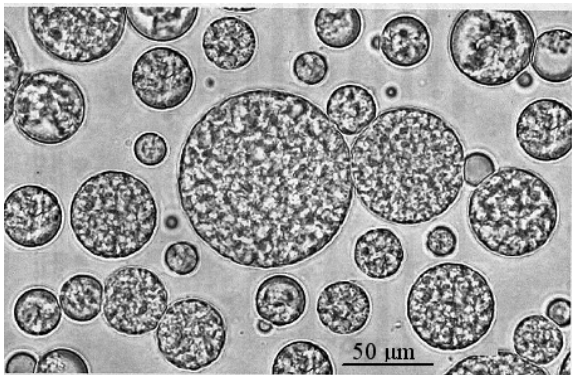


**Figure 1**. Photomicrography of the multiple emulsions immediately after preparation (magnification 1000x).

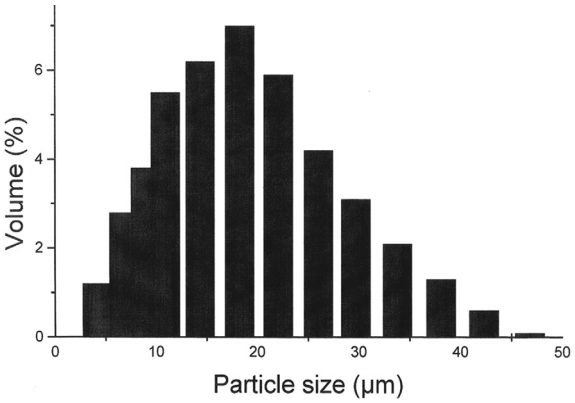


**Figure 2**. Distribution histograms of particle size of the multiple emulsion immediately after preparation.

The release curves of NTX hydrochloride from the W/O/W multiple emulsion diluted under hypo and iso-osmotic conditions, at 37 °C, in 480 min and 1 to 15 days of incubation are showed, respectively, in Figs. 3A and 3B. The release kinetics observed was in accordance with the swelling-breakdown mechanism. Thus, under iso-osmotic condition, no significant release was observed, whereas under hypo-osmotic condition NTX hydrochloride was released from the multiple emulsion in the first 120 min of the experiment. No significant release was observed under hypo and iso-osmotic condition in the 1 to 15 days of incubation period.

### *Tissue response to subcutaneous injection of W/O/W multiple emulsion*

No animal exhibited local adverse reaction following subcutaneous injection of the W/O/W multiple emulsion. However, cellular infiltration into injured tissue surrounding the injection site could be observed and a significant increase of about 10-fold and more in neutrophile granulocytes was observed 72 h after the injection (Fig. 4A). One week after injection of the formulation, the tissue response progressed from the initial inflammation and only a weak inflammatory response was observed which was indicated by the presence of a few polymorphs neutrophils and eosinophils (Fig. 4B). The histopathological studies of the excised tissues surrounding the injection site of the NTX hydrochloride solution alone did not show any inflammatory process up to 2 weeks. There was no capillary proliferation, fibroblast formation and monocyte infiltration even on the 14th day of administration which showed the absence of inflammation that could be due to the subcutaneous injection of the formulation in the studied period (Figure 4C). Host reaction was similar in every animal.

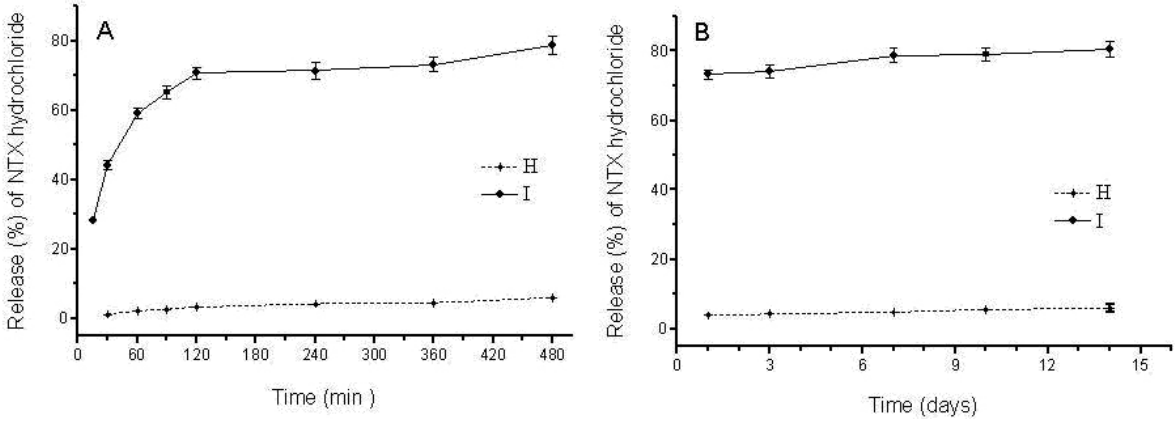


**Figure 3**. *In vitro* release profile of NTX hydrochloride from multiple emulsion in (H) hypo and (I) iso-osmotic condition. Drug release is reported in min (**A**) and incubation period (**B**). Values are presented as mean ± SD (n = 3).

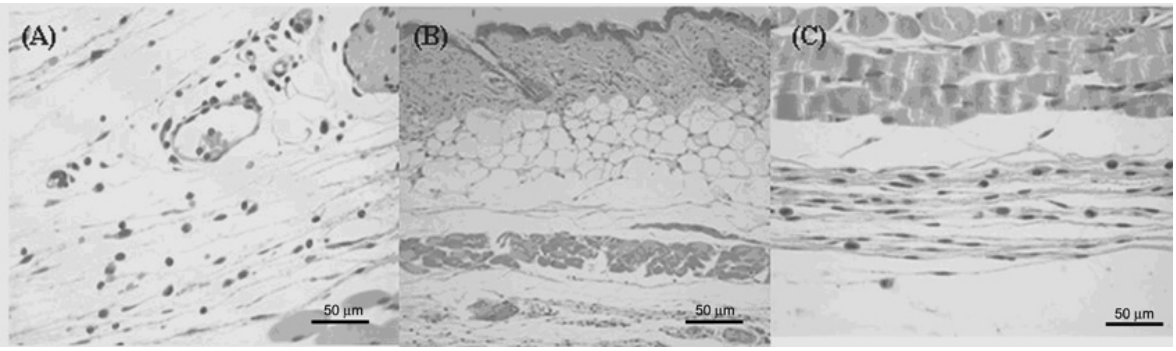


**Figure 4**. Photomicrographs of the histological sections of the subcutaneous tissue surrounding the site of injection of the multiple emulsion containing NTX hydrochloride after 3 (**A**), 7(B) and 14 (**C**) days. Hematoxylin-eosin staining was used. It can be observed that the initial inflammation showed in (**A**) started to disappear in (**B**) and completely disappears in (**C**). No other morphological changes in the structure of the tissue were visualized.

## DISCUSSION

This pilot study was designed to evaluate the preparation and the viability of a W/O/W multiple emulsion containing NTX hydrochloride obtained from pharmaceutical biocompatible materials. The emulsion was easily prepared by two-stage emulsification procedure. This method was used because it is considered to be a convenient and reliable procedure for encapsulation of water-soluble drugs [19]. The obtained system shows the relatively large (18.6 µm) and homogeneous particle sizes and high drug entrapment efficiency (97.7%). The advantages of this multiple emulsion, obtained at 20 °C, could be related to the properties of the lipophilic surfactant used (poloxamer 401) that provides well-defined film on the W/O globules [25]. The polymeric surfactants proved to be superior to the conventional non-ionic surfactants in maintaining the physical stability of the multiple emulsion [26,27].

The release kinetics study showed that in iso-osmotic condition there was no significant release of the drug. On the other hand, in hypo-osmotic condition NTX hydrochloride encapsulated was rapidly released from the multiple emulsion. In this case, the difference in the concentration gradient between the two aqueous phases caused the water flow from the external phase to the internal phase. This aqueous transport produced an increase in the internal globule size and consequent swelling of the oil globules which leaded to the release of the entrapped substance after breakdown of the oily membrane [21].

In the literature, two main release mechanisms are widely cited as possible methods of delivery for water-soluble drugs from W/O/W multiple emulsions *in vitro* release studies [28,29]. The release could be induced either by transport through the membrane or by membrane breakdown (following swelling or shearing). However, the NTX hydrochloride is hydrophilic enough (aqueous solubility 90 mg/ml at 20 °C to prevent its crossing through the oily membrane by simple diffusion. Moreover, the diffusion by micellar route could be excluded due to the nature of the surfactants employed, such as high molar mass. Thus, in the present study, the main release mechanism considered for the NTX hydrochloride encapsulated in the inner phase was a swelling breakdown phenomenon which followed dilution of the emulsion under hypo-osmotic conditions. The results obtained under hypo-osmotic condition showed a release time of approximately 120 min. After this, the presence of the NTX hydrochloride in the external aqueous phase result in reduction of the concentration gradient between the two aqueous phases with a consequent decrease in the osmotic aqueous flow and in the release mechanism. Taking into account the high osmolarity and the low volume of fluid in the subcutaneous tissue, the probable release mechanism for the NTX hydrochloride from multiple emulsion after administration by this route could be due to biodegradation of the system depot. On the basis of this consideration we suppose that the multiple emulsion could represent an alternative way for the administration of NTX. More studies are currently under investigation to evaluate bioavailability and pharmacokinetics of NTX hydrochloride entrapped in W/O/W multiple emulsion after subcutaneous administration.

Histopathological studies confirmed the biocompatibility of the developed W/O/W multiple emulsion containing NTX hydrochloride. On the 14th day after the administration of the formulation the sections showed no signs of inflammation or necrosis and were found normal when compared to control tissues. The absence of inflammatory response after 2 weeks can be explained by a complete biodegradation of the administered system.

**REFERENCES**


1. Yin, W., E.O. Akala & R.E.Taylor (2002) *Int. J. Pharm.* **244**: 9-19.
2. Costantini, L.C., S.R. Kleppner, J. McDonough, M.R. Azar & R. Patel (2004) *Int. J. Pharm.* **283**: 35-44.
3. O'Brien, C.P., L.A. Volpicelli & J.R. Volpicelli (1996) *Alcohol* **13**: 35-9.
4. Bartus, R.T., D.F. Emerich, J. Hotz, M. Blaustein, R.L. Dean, B. Perdomo & A.S. Basile (2003) *Neuropsychopharmacology* **28**: 1973-82.
5. Garbutt, J.C., H.R. Kranzler, S.S. O'Malley, D.R. Gastfriend, H.M. Pettinati, B.L. Silverman, J.W. Loewy & E.W. Ehrich (2005) *J. Am. Med. Assoc.* **293**: 1617-25.
6. Dean, R.L. (2005) *Front. Biosci.* **10**: 643-55.
7. Hulse, G.K., V. Stalenberg, D. McCallum, W. Smit, G. O'Neil, N. Morris & R.J. Tait (2005) *J. Control. Release* **108**: 43 – 55.
8. Ngo, H.T.T., D.E. Arnold-Reed, R.C. Hansson, R.J. Tait & G.K. Hulse (2008) *Prog. Neuropsychopharmacol. Biol. Psychiatry* **32**: 23-8.
9. Giannola, L.I., V. De Caro, G. Giandalia, M.G. Siragusa, C. Tripodo, A.M. Florena & G. Campisi (2007) *Eur. J. Pharm. Biopharm.* **67**: 425-33.
10. Alvarez-Fuentes, J., I.Caraballo, A. Boza, J.M. Llera, M.A. Holgadon & M. Fernández-Arévalo (1997) *Int. J. Pharm.* **148**: 219- 30.
11. Silva-Cunha, A., M. Chéron, J. L. Grossiord, F. Puisieux & M. Seiller (1998) *Int. J. Pharm.* **169**: 33-44.
12. Shively, M.L. (1997) *Pharm. Biotech.* **10**: 199-211.
13. Cournarie, F., M.-P. Savelli, V. Rosilio, F. Bretez, C. Vauthier, J.L. Grossiord & M. Seiller (2004) *Eur. J. Pharm. Biopharm.* **58**: 477-82.
14. Nakhare, S. & S.P. Vyas (1997) *Pharmazie* **52**: 224-46.
15. Khopade, A.J. & N.K. Jain (1999) *Pharmazie* **54**: 915-9.
16. Uno, T., T. Yamaguchi, X.K. Li, Y. Suzuki, H. Hashimoto, Y. Harada, T. Kimura, & T. Kazui (1997) *Lipids* **32**: 543-8.
17. Khopade, A.J. & N.K. Jain (1998) *Drug Dev. Ind. Pharm* **24**: 677-80.
18. Oh, I., Y.G. Kang, Y.B. Lee, S.C. Shin, C.K. Kim (1998) *Drug Dev. Ind. Pharm.* **24**: 889-94.
19. Okochi, H. & M. Nakano (2005) *Adv. Drug Deliv. Rev.* **45**: 5-26.
20. Lindenstruth, K. & B.W. Müller (2004) *Eur. J. Pharm. Biopharm.* **58**: 621-7.
21. Tedajo, G.M., S. Bouttier, J. Fourniat, J.L. Grossiord, J.P. Marty & M. Seiller (2005) *Int. J. Pharm.* **288**: 63-72.
22. Strickley, R.G. (2004) *Pharm. Res.* **21**: 201-30.
23. Silva-Cunha, A., J. L. Grossiord, F. Puisieux & M. Seiller (1997) *Int. J. Pharm.* **158**: 79-89.
24. Ursica, L., D. Tita, I. Palici, B. Tita & V. Vlaia (2005) *J. Pharm. Biomed. Anal.* **37**: 931-6.
25. Sela, Y., S. Magdassi & N. Garti (1993) *1er congrès mondial des emulsions,* Paris.
26. Tadros, T.F., M.C. Taelman & J.C. Dederen (1998) *"Multiple emulsions with polymeric surfactants",* in: "Multiple emulsions: structure, properties and applications", (J.L. Grossiord, M. Seiller, eds.) Editions de Sante, Paris, pp. 117–37.
27. Vasiljevic, D., J. Parojcic, M. Primorac & G. Vuleta (2006) *Int. J. Pharm.* **309**: 171-7.
28. Jager-Lezer, N., I. Terisse, F. Bruneau, S. Tokgoz, L. Ferreira, D. Clausse, M. Seiller & J.L. Grossiord (1997) *J. Control. Release* **45**: 1-13.
29. Grossiord, J.L. & M. Seiller (2001) *STP Pharm. Sci.* **11**: 331-9.