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RESEARCH ARTICLES

Pharmacokinetic Evaluation of Nalmefene PLGA Microspheres in Rats

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SUMMARY

Pharmacotherapies of insobriety is very limited till date. Few pharmacotherapies have been established as anti-craving drugs to reduce relapse risk or alcohol intake in alcoholism. The available bolus administration pharmacotherapy is also impeded by the higher rates of patient non-compliance, unwanted adverse reactions, and *fluctuating drug levels. A long-term drug delivery system would help overcome upon these limitations. The purpose of this work was to perform comparative pharmacokinetic evaluation* and *define the appropriate dosage regimen of different extended release nalmefene loaded PLGA biodegradable microspheres formulations prepared by O/O emulsification solvent evaporation method for the treatment of alcoholism. MSA, MSB* and *MSC achieved peak plasma concentration of 105. 80 ± 15. 30 ng/mL, 164. 29 ± 32. 27 ng/mL* and *262. 94 ± 48. 94 ng/mL in 72 hr, 12 hr* and *12 hr, respectively* and *plasma concentrations sustained upto 720-1080 hr. The plasma exposure (AUClast) achieved by SC injection, IV injection, MSA, MSB* and *MSC nalmefene formulations are 442. 38 ± 64. 31, 613. 86 ± 75. 13, 57553. 28 ± 8320. 60, 48878. 81 ± 9603. 06* and *52805. 75 ± 9828. 14 hr. ng/mL, respectively. The optimum predicted dosing regimen for each of these formulations would be as 21-day, 7-day* and *14-day dosing for MSA, MSB* and *MSC formulation, respectively. The results of the study demonstrated the feasibility of long term delivery of nalmefene using PLGA biodegradable microspheres by providing a relatively constant nalmefene plasma concentration for at least one to two months in rats.*

Key Words: *Nalmefene, PLGA, Microspheres, Sustained Release, Pharmacokinetics, Formulation*

Sıçanlarda Nalmefen Yüklü PLGA Mikroküre lerinin Farmakokinetik Olarak Değerlendirilmesi

ÖZET

*Alkolizmin farmakoterapisi günümüzde oldukça sınırlıdır. Alkolizmde tekrar etme riskini engellemek veya alkol alımı isteğini azaltıcı birkaç farmakoterapik ilaç belirlenmiştir. Hasta uyuncunun düşük olması, istenmeyen yan etkiler ve ilaç seviyesindeki dalgalanmalar mevcut bolus farmakoterapi uygulamalarını engellenmektedir. Uzatılmış etkili ilaç uygulama sistemi, bu kısıtlamaların üstesinden gelmeye yardımcı olacaktır. Bu çalışmanın amacı, karşılaştırmalı farmakokinetik değerlendirme yapmak ve alkolizmin tedavisi için yağ/yağ emülsiyon çözücü uçurma yöntemi ile hazırlanan farklı uzatılmış salımlı nalmefen yüklenmiş biyolojik olarak parçalanabilir PLGA mikroküre formülasyonlarının uygun dozaj formunu geliştirmektir. MSA, MSB ve MSC sırasıyla 72 saat, 12 saat ve 12 saatte 105. 80 ± 15. 30 ng / mL, 164. 29 ± 32. 27 ng / mL ve 262. 94 ± 48. 94 ng / mL plazma pik konsantrasyonuna ulaşmıştır ve plazma konsantrasyonları 720-1080 saate kadar uzatılmıştır. Nalmefen formülasyonlarının SC enjeksiyonu, IV enjeksiyonu, MSA, MSB ve MSC uygulamaları ile elde edilen plazma eğri altında kalan alan(AUCson) sırasıyla 442. 38 ± 64. 31, 613. 86 ± 75. 13, 57553. 28 ± 8320. 60, 48878. 81 ± 9603. 06 ve 52805. 75 ± 9828. 14 ng. saat/mL'dir. Bu formülasyonların her biri için optimum öngörülen doz uygulaması, MSA, MSB ve MSC formülasyonları için sırasıyla 21 günlük, 7 günlük ve 14 günlük doz olacaktır. Çalışmanın sonuçları sıçanlarda en az bir ila iki ay süreyle nispeten sabit bir nalmefen plazma konsantrasyonu sağ*land*ığını, PLGA biyolojik olarak parçalanabilir mikroküreleri kullanarak nalmefenin uzun süreli verilmesinin uygulanabilirliğini göstermiştir.*

Anahtar kelimeler: *Nalmefen, PLGA, Mikroküreler, Sürekli Salım, Farmakokinetik, Formülasyon*

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INTRODUCTION

Management of alcohol dependence is a major mental health problem in India and throughout the world. It's estimated that of Indian population 5-7% abuse alcohol and 10-20 million people are in need of treatment. About 1. 2% of total deaths are accounted to alcohol dependence (Grover S, 2007). It has also been seen that only 8-10% of total alcohol dependent European patients and 25% American patients are treated in specialty settings (Dawson DA, 2005 and Rosner S, 2005). As on date with a better understanding of the basic neurobiological components of alcohol dependence, we have pharmacological agents targeted at improving drinking behavior, enhancing abstinence and preventing relapse as well as reducing the amount of alcohol people drink when they relapse. There are currently three US FDA approved medications for the relapse prevention of alcohol dependence. These are Disulfiram, Naltrexone and Acamprosate. Among these drugs disulfiram was the only pharmacological treatment for alcohol dependence available in the USA for many years, despite high rates of severe adverse drug reactions, drinking relapse, and medication (Fuller RK, 1986). Later, Naltrexone was approved in 1994 as a non-aversive prescription drug for alcohol dependence though benefits of naltrexone in recent studies are modest. Intolerable nausea (Croop R, 1997 and Luquiens A, 2014) and dose-dependent hepatotoxicity limit naltrexone use. Among the available treatments, Nalmefene approval was a milestone in pharmacotherapy of alcohol dependence. It is a μ receptor selective opioid antagonist similar to naloxone in structure and pharmacology. It also possesses the antagonistic properties at δ-receptor and partial agonistic properties at the k-receptor (Dixon R, 1987 and Bart G, 2005). On comparision with naltrexone, it has the advantages of longer half-life (~10 h), greater oral bioavailability and lower dose-dependent liver toxicity (Costantini LC, 2004 and Gual A, 2014).

Current available pharmacotherapies could not achieve the expected success in managing alcohol dependence due to several factors such as a) poor compliance to regimen by patients b) fluctuations in concentrations of drug levels in blood c) adverse effects at the doses required for clinical efficacy. These factors may result in the interruption of therapy or premature discontinuation (Costantini LC, 2004) and thus lead to the failure of the pharmacological treatments.

To improve upon the limitations posed by the available pharmacotherapies a long term delivery system would be of benefit. Accordingly, Costantini *et al.* prepared an implantable ethylene vinyl acetate (EVA) rod containing nalmefene for sustained release (Costantini LC, 2004). However, it could not be of practical utility as the EVA rod non erodible in the invivo system and hence needs to be removed from the body once it runs out of the drug. Therefore there is a need of a biodegradable injectable delivery system which may solve the non-erodibility issue faced with EVA rods. A good amount of research on drug delivery by biodegradable polymers (Gultekin, 2013) has been conducted and reported in the literature that they are able to extend the duration of drug action, reduce the dosing frequency and thus improve upon the patient compliance (Hickeya T, 2002). Dose and certain adverse effects could be diminished due to the stable blood drug levels achieved by the microspheres (Wu, 2006). Another advantage is that there is no need of implantation and removal of the microspheres by surgical operation during the period of drug delivery. Poly (lactic-co-glycolic acid) (PLGA), as a biodegradable polymer has been approved by US FDA for in vivo use and has been employed in several of commercially marketed parenteral formulations (e. g. Zoladex, Trelstar, Vivitrol, Lupron Depot and Risperdal Consta). As it's demonstrated that PLGA has a good biocompatibility and able to deliver the drug over longer duration of time it could be utilised as a drug delivery system for delivering nalmfene for the treatment of alcohol dependence.

A study was conducted by Wu et al (Wu, 2006) wherein the PLGA microspheres of nalmefene were developed without any further studies on the in vitro release mechanism and in vivo performance of nalmefene microspheres. In a study (Xie *et al,* 2015) the authors prepared PLGA microspheres containing nalmefene following the method proposed by (Wu, 2006) using oil-in-oil (O/O) emulsification solvent evaporation method and evaluated the *invitro* and *invivo* performance of the microspheres. They have concluded that injectable PLGA microspheres containing nalmefene can be used as a promising long-term treatment option for alcohol dependence.

Taking the lead from (Xie *et al,* 2015), in our current paper, we wanted to evaluate a slightly different composition of PLGA and Nalmefene which would result in different microsphere formulations and compared their pharmacokinetics in the Male Sprague Dawley rats with nalmefene solution formulations administered by intravenous and subcutaneous routes.

Our main objective was to evaluate and compare the pharmacokinetics of

A) Different PLGA and Nalmefene composition

microsphere formulations administered by subcutaneous (SC) route,

B) Nalmefene solution formulations administered by intravenous bolus (IV) and subcutaneous (SC) routes

and to define the optimum dosage regimen for each of the microsphere formulations following multiple dose administration.

MATERIALS And **METHODS**

PLGA (molecular weight 20000, 45000 and 74000Da, lactide/glycolide ratio, 50/50 and 75/25) were purchased from the Sigma Aldrich, St. Louis, MO, USA in their hydrophilic forms (carboxylic acid end group). Nalmefene Hydrochloride (> 99% purity) were supplied by Alkem Drugs Pharmaceutical Company (Baddi, India). Nalbuphine (99. 6% purity), the internal standard, Liquid paraffin, dichloromethane (DCM) and acetonitrile (AN) was purchased from Sigma (St. Louis, MO, USA). Tween-80 was supplied by Fisher Scientific (Hongkong, China) Ltd. All other materials or solvents were of reagent or analytical grade.

Preparation of microspheres

Microsphere-A (MSA):

An O/O emulsification solvent evaporation approach based on the description of (Xie *et al.* 2015) was applied to prepare the microspheres. Briefly, 180 mg of PLGA and 15 mg of nalmefene were added and dissolved in 1 mL of Dichloromethane –Acetonitrile (DCM-AN) solvent (1: 1, v/v). Then, the mixture was poured quickly into 20 mL of liquid paraffin containing Span-80 (1. 5%, w/v) as emulsifier, and emulsified through a propeller stirrer (SXJQ-1, Zhengzhou, China) at 650 rpm (rotations per minute) for 10 min at the temperature of 25°C. To evaporate the organic solvent in the O/O emulsion, the stirring speed was changed to 450 rpm and kept for 10 h. By filtering through a filter-paper, the solidification microspheres were gathered, rinsed 3 times with 15 mL hexane and then washed with 20 mL deionized water. Finally, the collected microspheres were dried at a vacuum chamber under room temperature.

Microsphere –B and C (MSB and MSC):

An O/O emulsification solvent evaporation approach method was followed (D'Souza S, 2014). The two formulations prepared were 54 kDa PLGA, 75:25 lactide:glycolide (Formulation MSB) and 65 kDa PLGA, 75:25 lactide:glycolide (Formulation MSC). Briefly, 3 g of PLGA or their blends were dissolved with 1. 5 g of nalmefene in 15 mL of dichloromethane (DCM), the external phase was 0. 5% (w/v) aqueous polyvinyl alcohol (PVA) solution. First, the organic phase was emulsified with 1500 mL of 0. 5% aqueous PVA solution (2000 rpm for 4 min) in a homogenizer at room temperature. Second, the dispersion was stirred with a Silverson L4R mixer (Silverson machines, MA, USA) at 5000 rpm for 4 h at room temperature to harden the microspheres. The microspheres were collected by filtration, washed extensively three times with deionized water. 0. 5 mL of 15% mannitol aqueous solution was added to prevent the microspheres from aggregation. After freeze drying, the microspheres were weighed and stored at 4°C. Briefly, the three formulations details prepared by using different blends of PLGA polymer were given in Table-1.

Table 1: Formulation details of MSA, MSB and MSC

Formulation	MSA	MSB	MSC
MW	20kDa	54kDa	65kDa
PLGA type	75:25	75:25	75:25
Drug loading	6. 87± 3. 56%	6. 54± 1. 25%	7. 14± 0. 56%
Encapsulation efficiency	79. 45 ± 4. 63	75. 23 ± 3. 25	82. 35 ± 5. 95
Dose (mg/kg)	90	90	90

In vitro release assay

The in vitro release studies of nalmefene from the microspheres were carried out in 30-mL cylindrical tubes containing 25 mL of phosphate buffer solution (PBS, 0. 1 M, pH 7. 4), 0. 02% sodium azide (w/v) and 0. 5% sodium dodecyl sulphate (SDS, w/v). In each tube, 10 mg of prepared microspheres were added. The tubes were incubated in a waterbath at 37 ± 0. 5°C and vibrated horizontally at a speed of 72 rpm [Mason BJ, 1999 and Wu J, 2013]. The nalmefene concentrations were assayed by the HPLC.

Drug loading and **entrapment efficiency**

Drug loading and Entrapment Efficiency of MSA, MSB and MSC formulations was determined according to the procedure described by (Xie X,

2014). Briefly, accurately weighed 25 mg nalmefene microspheres were dissolved in 2 mL of an aqueous acetonitrile solution (9: 1, v/v) and followed by a 10 fold dilution with 0. 001 mol/L HCl. The resulting solution vortexed for 2 min and then kept undisturbed for 5 min. After centrifuged with a speed of 10, 000 rpm for 10 min, the supernatant was collected and its nalmefene concentration was analysed (Chou JZ, 1993) by a high performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, USA). The HPLC consisted of a pump and a ultra-violet and visible light (UV-Vis) detector at wavelength of 284 nm. A reversed phase C18 column (5 µm, 4. 6 mm × 250 mm, Agilent technologies, USA) was used at 25°C. A mixture of KH_2PO_4 aqueous solution (pH 4. 0; 0. 02 M), methanol and triethylamine (60: 40: 0. 2, v/v/v) was used as the mobile phase at the flow rate of 1 mL/min. The injection volume was 20 µL.

The drug loading (DL, %) and encapsulation efficiency (EE, %) were calculated by the following equations:

$$DL(\%) = \frac{Drug\ found\ in\ microspheres}{microspheres\ weight} x100$$

$$EE(\%) = \frac{Drug\ found\ in\ microspheres}{Drug\ added} x100$$

Pharmacokinetics study

This study was conducted in compliance with Institutional Animal Ethics Committee (IAEC) requirements. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) of JVR Bio Life Sciences (IAEC/2012/1006076). All the ethical practices as laid down in the Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA) guidelines for animal care was followed during the conduct of the study. Further, procedures used in this protocol was designed to conform to the accepted practices and to minimize or avoid risk of causing pain, distress or discomfort to the animals.

Briefly, a total of 30 male Sprague-Dawley rats (220 ± 20 g, 6 weeks) were randomly divided into five groups each of 6 rats. In the group-1, nalmefene solution (diluted with sterile water) was dosed by Intravenous (IV) Bolus route through tail vein at the dose of 5 mg/kg. Group-2 animals were dosed by subcutaneously (SC) injected at the dose of 5 mg/kg. In the group-3, 4 and 5, the MSA, MSB and MSC microspheres were UV (Ultraviolet light) sterilized and then they were dispersed in 2 mL water for injection (containing 0. 05% Tween-80 and 0. 5% Sodium Carboxy Methyl Cellulose) before the administration then the microsphere suspensions (90 mg / kg) were subcutaneously injected into the back of the rat by a 21 gauge needle. The dosing volume was maintained as 5mL/kg for both IV and SC routes

Blood samples of about 0. 24 mL were collected from all the animals at the designated time points through retro orbital plexus under brief isoflurane anaesthesia. The blood samples were collected and transported into heparin tubes . The blood sampling time points for different groups are as follows, For group-1 0 hr (predose), 0. 083, 0. 17, 0. 5, 1, 2 and 3 hr post dose, For group-2 at 0 hr (predose), 0. 03, 0. 08, 0. 25, 0. 5, 0. 75, 1, 2, 3, 5, 7, 12, 24, 30 hr post dose, for the group-3 at 0 hr (predose), 0. 5, 1, 2, 3, 5, 7, 14, 21, 28, 35 days for the group-3, For group 4 and 5 at 0 hr (predose), 0. 5, 1, 4, 8, 15, 20, 30 and 45 days, respectively. Plasma samples were obtained by centrifuging (4°C) the blood samples at 4, 000 rpm for 10 min, then collected the plasma and frozen them at -70°C until analysis.

LC MS/MS Analysis:

Chromatographic analysis to determine the plasma nalmefene concentration was performed using an Agilent 1200 series LC (Liquid Chromatography) system coupling with tandem mass spectrometry (LC-MS/MS, Agilent Technologies, Santa Clara, CA, USA) as described by Fang et al (Fang WB, 2005), the LC column was a YMC ODS-AQ (5-µm, 120 A, 2. 0 x 100 ram, Waters Corporation, Milford, MA). The mobile phase was 75% of 0. 1% formic acid in water and 25% of methanol with a flow rate of 0. 2 mL/min. Autosampler temperature was set at 15°C and column temperature was set at 30°C Injection volume was 10 µL. Run time was 8 min.

MS/MS mass spectrometer, triple quadrupole, AB-Sciex model API 5000 equipped with atmospheric pressure electrospray ionization interface was used for analysis. It was equipped with Data system based on Dell computer with Windows 7 operating system using AB-Sciex software Analyst 1. 6. 1 for data acquisition and processing. The HPLC was interfaced to the Mass Spectrometer by means of an ESI manifold. The instrument was operated under selective reaction monitoring mode. Capillary temperature was 250 ESI spray voltage was set at 4. 5 kV. High purity N2 was used for both sheath gas and auxiliary gas which were set at 60 psi and 10 flow units, respectively. High purity Ar (3 mTorr) was used for collision gas. The Xcalibur software was used for the setup and operation of sequence lists and LC Quan (within the Xcalibur software) was used for batch quantitation. The m/z 340 (MH +) to 322 selected reaction monitoring transition was used to analyze nalmefene. The m/z 358 (MH +) to 340 transition was

used to analyze nalbuphine.

Sample Preparation:

The sample preparation method was according to (Fang WB, 2005). Nalmefene concentration was quantified by LC–MS/MS, using nalbuphine as an internal standard. Briefly, a 100 µL plasma sample mixed with 50 µL of internal standard, (0. 1 ng/mL in Milli-Q water) were added followed by 100 µL of concentrated ammonium hydroxide to increase the pH of the plasma (> 10). This was followed by addition of 4 mL of n-butyl chloride/acetonitrile (4:1) mixture. Tubes were then capped tightly and placed on a reciprocating tube rocker at low speed for 30 min. They were then centrifuged at 2400 rpm for 10 min, and the organic layer was carefully transferred into 13 x 100 mm silanized culture tubes. The organic solvent was evaporated off under 15 psi air at 40^0 C in a Zymark Turbo Vap evaporator. The residues were reconstituted in 500 µL of 0. 1% formic acid in water/ methanol (9:1) mixture, and 20 µL was transferred to autosampler vials. The calibration curve range selected was 0. 5-5000 ng/mL.LL0Q was 0.5 ng/mL

Pharmacokinetic Data Analysis:

The pharmacokinetic parameters of nalmefene were calculated using the non-compartmental analysis (NCA) tool of the Phoenix® software (Version 6. 4). The area under the concentration time curve (AUC_{last} and AUC_{inf}) was calculated by linear trapezoidal with linear interpolation rule. Peak plasma concentration (C_{max}) and time for the peak plasma concentration (T_{max}) were observed values. The C^0 (back extrapolated concentration at time zero) was estimated following intravenous bolus dose administration by back extrapolating from the first two concentration values. The elimination rate constant value (K_{el}) was calculated by linear regression of the log-linear terminal phase of the concentration-time profile using at least 3 declining concentrations in terminal phase with adjusted $R^2 > 0.8$. The terminal half-life value ($T_{1/2}$) was calculated using the equation $0.693/K_{el}$. The absolute bioavailability of different formulations with respect to intravenous formulation was calculated and reported.

Selection of dosage regimen:

To predict the in vivo profile of nalmefene PLGA microspheres for a prolonged duration, plasma levels through 4 doses for all formulations were simulated using the superposition principle using Non Parametric Superposition (NPS) tool of Phoenix® software (Version 6. 4).

RESULTS And **DISCUSSION**

The drug loading of the prepared microsphere formulations MSA, MSB and MSC was 6.87± 3.56%, 6.54± 1.25% and 7.14± 0.56%, respectively and the encapsulation efficiency (n=3) was 79.45 ± 4.63%, 75. 23 ± 3. 25% and 82. 35 ± 5. 95%, respectively.

The in vitro drug release plot (cumulative release versus time) of nalmefene loaded microspheres was demonstrated in Fig 1. During initial 24hr approximately 8% of the drug from MSA and 4% of the drug from MSB and MSC formulations was released, then by day 14 approximately 61% of the drug from MSA and 49% and 55% of the drug from MSB and MSC formulations, respectively was released and by day 21 approximately 78% of drug from MSA, 81% and 79% was released. By the day 27, 87% of drug from MSA, and by day 28 101% and 97% of drug was released from MSB and MSC formulations, respectively.

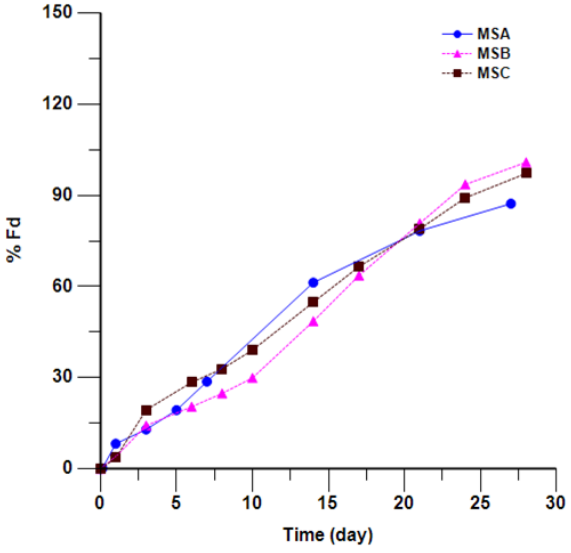

Figure 1: Mean in vitro release profile of nalmefene loaded microspheres. In 0. 1 M PBS (pH 7. 4) at 37°C (n = 6).

Within the same copolymer (75:25), a comparison of release profiles for Formulations MSA, MSB and MSC revealed slightly faster release for the higher molecular weight Formulation MSC. This was presumably due to the higher drug load (i. e. , higher drug to polymer ratio). On day-3 MSC formulation exhibited higher release compared to MSB and MSA and it sustained higher release until day 8. This in-vitro behaviour translated into in-vivo behaviour of MSC formulation as a result of which higher initial burst release was observed.

The mean (±SD) plasma profiles of nalmefene following intravenous and subcutaneous administration was presented in figure-2 followed by mean(±SD) plasma profiles of nalmefene following subcutaneous administration of nalmefene microspheres is presented in figure-3. The plasma drug concentration of nalmefene following single dose SC injection reached its maximum value (C_{max}) of 685. 24 ± 108. 83 ng/mL in 2 min (T_{max}).

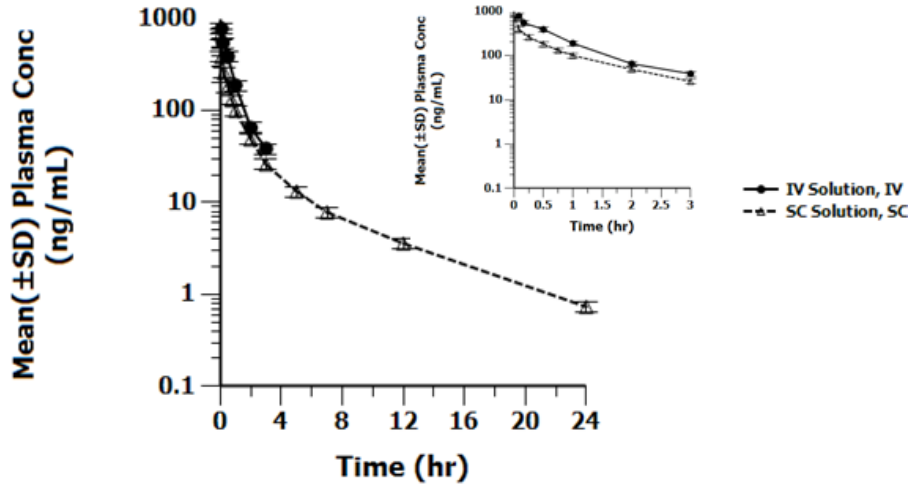

Figure 2: Mean (±SD) Plasma Concentration vs Time Profile of Nalmefene following intravenous and subcutaneous administration (Dose: 5 mg/kg) of solutions formulation in Male Sprague Dawley Rats (n=6)

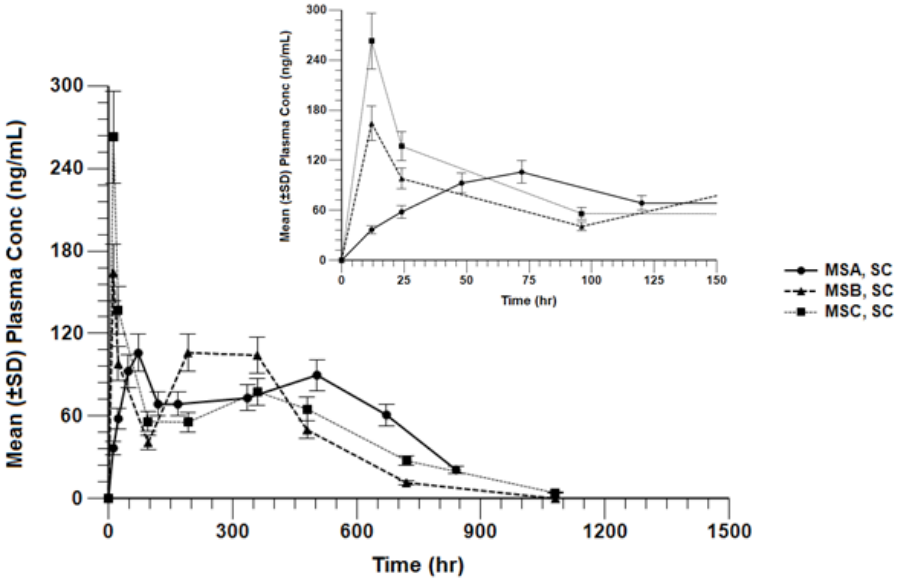

Figure 3: Mean (±SD) Plasma Concentration vs Time Profile of Nalmefene following subcutaneous administration (Dose: 90 mg/kg) of MSA, MSB and MSC Nalmefene Microsphere formulation(s) in Male Sprague Dawley Rats (n=6)

Among the nalmfene microsphere formulations, MSA microsphere formulation achieved peak mean plasma concentration (C_{max}) of 105.80 ± 15.30 ng/mL at 72 hr; then the drug concentration declined and remained at a value of about 70 ng/mL from 120 hr to 336 hr; after that, the plasma concentration slightly increased and achieved second peak of 89.74 ± 12.97 ng/mL at 504hr; following that, the plasma concentration dropped slowly and reached 20 ng/mL at the 840 hr. The first high plasma drug concentration of nalmefene in the earlier five days was due to the initial fast drug release from the microparticles

For MSB microsphere formulation, the plasma nalmefene concentration approached its C_{max} of 164.29 ± 32.27 ng/mL at the 12 hr (0.5 day); then, the drug concentration decreased markedly to 40 ng/mL at day 4; after that, the plasma concentration rose gradually and exhibited its second peak value (106.03 ± 10.53 ng/mL) at day 8; following that, the plasma drug concentration dropped slowly and reached <10 ng/mL after the day 30.

For MSC microsphere formulation a similar trend was observed to MSB the plasma nalmefene concentration approached its C_{max} of 262.94 ± 48.94 ng/

mL at the 12 hr (0.5 day); then, the drug concentration decreased markedly to 55 ng/mL at day 8; after that, the plasma concentration rose gradually and exhibited its second peak value (77.66 ± 19.45 ng/mL) at day 15; following that, the plasma drug concentration dropped slowly and reached <10 ng/mL at the day 45. MSB and MSC microsphere formulations achieved a faster higher peak plasma drug concentration due to the greater and quick initial burst release of PLGA microspheres compared to MSA formulation.

The plasma exposure (AUC_{last}) achieved by SC injection, IV injection, MSA, MSB and MSC nalmefene formulations are 442.38 ± 64.31, 613.86 ± 75.13, 57553.28 ± 8320.60, 48878.81 ± 9603.06 and 52805.75 ± 9828.14 hr. ng/mL, respectively.

In summary, microsphere formulations MSA, MSB, and MSC depict similar in vivo behavior characterized by a high initial burst which can be attributed to surface associated drug. As the initial burst was completed, the circulating drug concentrations starts depleting which leads to a trough that was followed by a slow sustained release of drug from the PLGA matrix until values diminished. The initial burst phenomenon with reduced C_{max} compared to delivering a bolus dose is an important advantage associated with PLGA delivery systems which is particularly is desirable in certain therapeutic regimens, especially those involving long term therapy.

The absolute bioavailability (% F_{abs}) of SC injection formulation was 72%. It was expected that all three PLGA microsphere formulation would achieve >100% bioavailability due to their sustained plasma drug levels continuously for longer duration of time. Relative bioavailability of MSB and MSC was 92% and 88%, respectively with respect to MSA formulation. The elimination half-life of nalmefene by iv administration was very quick and rapid with 0.72 hr compared to 5.02 hr after SC administration whereas in case of the three MSA, MSB and MSC microsphere formulations it was 159.58 hr, 113.41 hr and 147.85 hr, respectively.

The mean pharmacokinetic (PK) parameters of SC injection, IV injection, MSA, MSB and MSC nalmefene formulations are listed in Table 2. Compared with IV and SC drug solution formulations, the values of T_{max}, AUC_{last}, $AUC_{0\text{-}inf}$ and $T_{1/2}$ of PLGA nalmefene microsphere formulations were all significantly higher, which suggests that the nalmefene microspheres formulations by different compositions performed similar to our expectation of a sustained release feature.

Table 2: Mean (± SD) Pharmacokinetic Parameters of Nalmefene following administration of intravenous, subcutaneous solutions and subcutaneous Nalmefene microspheres suspension formulations in Male Sprague Dawley Rats (n=6)

Route/ Formulation	Dosing (mg/kg)	T_{max} [@] (hr)	C_{max} (ng/mL)	AUC_{last} (hr*ng/mL)	$AUC_{0\text{-}inf}$ (hr*ng/mL)	$AUC_{0\text{-}3}$ (hr*ng/mL)	$AUC_{0\text{-}720}$ (hr*ng/mL)	$T_{1/2}$ [@] (hr)	Abs Bio %F	Rel Bio %F
IV Bolus/ Solution	5	NA	1116. 49* ± 172. 63	613. 87 ± 75. 13	653. 94 ± 75. 13	NA	NA	0. 72	100	NA
SC/ Solution	5	0. 03	685. 24 ± 108. 83	442. 38 ± 64. 31	447. 71 ± 65. 08	327. 465 ± 47. 697	NA	5. 02	72	NA
SC / MSA	90	72	105. 80 ± 15. 30	57553. 26 ± 8320. 60	62353. 93 ± 9014. 65	NA	53338. 54 ± 7711. 27	159. 58	>100	NA
SC / MSB	90	12	164. 24 ± 32. 27	48878. 81 ± 9603. 06	50766. 21 ± 9973. 87	NA	48878. 81 ± 9603. 06	113. 41	>100	92
SC / MSC	90	12	262. 94 ± 48. 94	52805. 75 ± 9828. 14	53725. 97 ± 9999. 41	NA	47127. 12 ± 8771. 24	147. 85	>100	88

$AUC_{0\text{-}3}$: Partial $AUC_{0\text{-}3}$; Abs Bio: Absolute Bioavailability (%); T_{max} and $T_{1/2}$ [@] is given as median values;*C^0 value ; NA: Not Applicable

In a similar study (14) the authors have evaluated the nalmefene loaded PLGA microspheres and reported the (C_{max}) peak plasma concentration of 111.42 ± 34.21 ng/mL with T_{max} of 76 hr post dosing and exposure (AUC_{last}) of 36720.45 ± 3536.16 hr. ng/mL. These values were in line with the values reported by us in our study. They did not study the absolute bioavailability of subcutaneous solution administration whereas we have observed 72% absolute bioavailability of SC injection in our study.

The main adverse effect exhibited by patients after IV bolus administration of nalmefene was nausea and it is related to the high peak plasma concentration (1116.49 ± 172.63 ng/mL) achievement (Xie *et al,* 2015). Therefore nausea as an adverse effect and requirement for daily administration of tablets or injections could also contribute to patient non-compliance which are some of the main hindrance factors to the long term treatment regimen. A recent study conducted by Rosner et al (Rosner *et al.*, 2015) on alcoholics receiving pharmacological and psychological treatment showed that the mean duration of abstinence is 154 days which provides window of greatest opportunity for pharmacological intervention. In addition to that the highest risk of relapse is during the first months after cessation of drinking and the percentage of patient non-compliance increases over time particularly between 2 and 6 months (Rosner *et al.*, 2015).

The factors leading to patient non-compliance could be overcome by using the PLGA microspheres technology for achieving lower peak plasma concentrations without compromising the efficacy and providing relative constant blood drug concentration in the long-term pharmacotherapy. In the current study all the factors listed above have been achieved by the three microsphere formulations MSA, MSB and MSC with peak plasma concentration of 105. 80 ± 15.30 ng/mL, 164.29 ± 32.27 ng/mL and 262.94 ± 48.94 ng/mL in 72 hr, 12 hr and 12 hr, respectively and the sustained plasma concentrations which lasted upto 720-1080 hr.

The reported efficacious oral dose for treating insobriety was 10-80 mg nalmefene/ day in clinical studies (Mason BJ, 1999). Based on this fact the target ratio for maintaining safety in humans was 8 (highest dose/ lowest dose) and considering the linear pharmacokinetics of nalmefene in humans it's assumed that the safety factor of 8 to be considered between peak plasma and maintenance plasma concentrations. Applying these assumptions to evaluate the clinical relevance of the microspheres formulations for MSA, MSB and MSC the peak plasma concentration was 105.80 ± 15.30 ng/mL, 164.29 ± 32.27 ng/mL and 262. 94 ± 48.94 ng/mL, respectively with maintenance plasma concentrations hovered around 70 ng/mL, 100 ng/mL and 60 ng/mL, respectively and safety ratio would be 1.5x, 2.75x and 2.6x, respectively. As safety ratio of microsphere formulations in rats was 1.5, 2.75 and 2. 6 smaller than 8, the peak plasma concentration that might produce by this depot formulation may be safe in human, however this has to be confirmed in studies further

To determine the optimal dosage regimen for the microspheres, simulations was performed using the concentration time data generated from a single dose which then was extrapolated to a multiple dosing scenario using the principle of superposition. For Formulations MSA, MSB and MSC, different dosing regimens such as 7-day, 14-day, 21-day and 28-day was attempted (Figure 4). Once again, a pulsatile release profile is observed primarily due to the initial burst observed with all the formulations. The half-life of MSA, MSB and MSC formulations calculated by NCA resulted in approximately ~ 160 hr (7 day), 113 hr (5 day) and 148 hr (7 day). Based on the concentration vs time profile the initial and second peak were observed at day 3 and 21 for MSA, at day 0.5 and 8 day for MSB and at 0.5 and 15 for MSC formulations, respectively, therefore the optimum dosing regimen for each of these formulations would be as 21-day, 7-day and 14-day dosing for MSA, MSB and MSC formulation, respectively. However we have simulated the plasma concentration profiles following multiple dosing for each of the formulations using all the 4 dosing regimens.

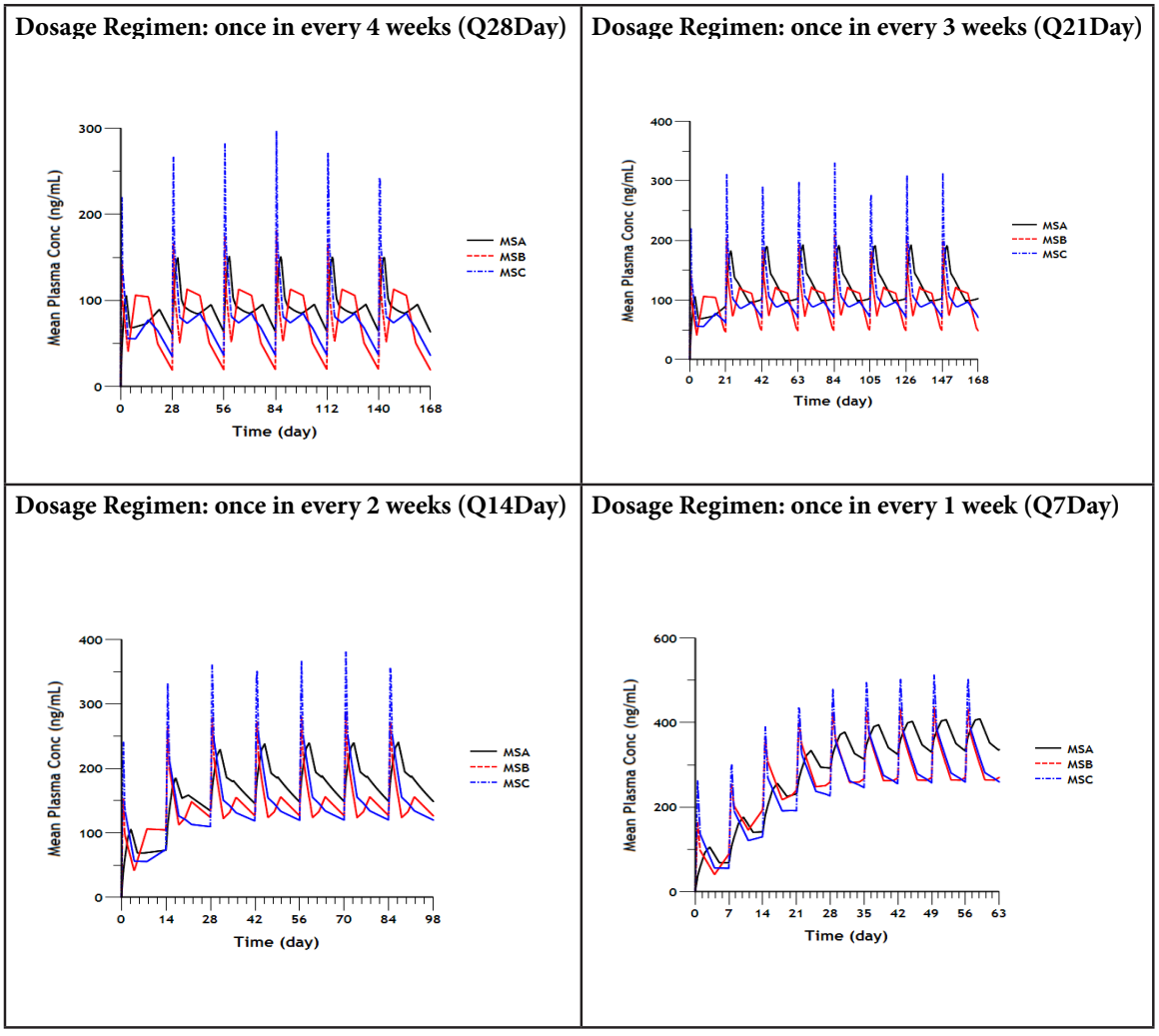

Figure 4: Predicted Mean Plasma Concentration vs Time Profile of Nalmefene following multiple subcutaneous administration (Dose: 90 mg/kg) of MSA, MSB and MSC Nalmefene Microsphere formulation(s) in Male Sprague Dawley Rats (n=6)

The local irritation and muscle stimulation tests performed (Xie, 2015) in rabbits demonstrated that the nalmefene microspheres could be used for subcutaneous or intramuscular administration, without causing any permanent damage to the skin or muscle, so based on this report it was assumed that PLGA loaded nalmefene microspheres prepared by similar method and composition would not cause any of these unwanted effects and could be comfortably developed for further dose administration

CONCLUSION

In our paper, the prepared nalmefene injectable PLGA microspheres provided the much required sustained plasma concentrations over 35-45 days which is much needed for treating disorders that require strict compliance such as insobriety. Therefore for the long-term treatment of alcohol dependence the nalmefene loaded PLGA microsphere extended release formulation may be of benefit.

Note: All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of Interest disclosure:

Harish Kaushik Kotakonda, Malothu Nagulu and Yellu Narsimha Reddy declare that they have no conflict of interest.

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