Formulation and optimisation of novel transfersomes for sustained release of local anaesthetic

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Abstract

Objective: To investigate the effect of formulation parameters on the preparation of transfersomes as sustained-release delivery systems for lidocaine and to develop and validate a new HPLC method for analysis.
Method: Taguchi design of experiment (DOE) was used to optimise lidocaine-loaded transfersomes in terms of phospholipid, edge activator (EA) and phospholipid:EA ratio. Transfersomes were characterised for size, polydispersity index (PDI), charge and entrapment efficiency (%EE). A HPLC method for lidocaine quantification was optimised and validated using a mobile phase of 30%v/v PBS (0.01M):70%v/v Acetonitrile at a flow rate of 1ml/min, detected at 255nm with retention time of 2.84 minutes. The release of lidocaine from selected samples was assessed in-vitro.
Key findings: Transfersomes were 200nm in size, with PDI~0.3. HPLC method was valid for linearity (0.1-2mg/ml, R^2 0.9999), accuracy, intermediate precision and repeatability according to ICH guidelines. The %EE was between 44-56% and dependent on the formulation parameters. Taguchi DOE showed the effect of factors were in the rank order: lipid: EA ratio >EA type >lipid type. Optimised transfersomes sustained the release of lidocaine over 24h.
Conclusion: Sustained-release, lidocaine-loaded transfersomes were successfully formulated and optimised using a DOE approach and a new HPLC method for lidocaine analysis was developed and validated.

Keywords: Transfersomes, design of experiment, local anaesthetic, sustained release, HPLC

1. Introduction

Lipid-based vesicles such as liposomes, niosomes, transfersomes and proliposomes provide several advantages in comparison to polymeric and non-vesicular systems [1-5]. A major advantage is their ability to deliver both hydrophilic and hydrophobic pharmacologically active drugs, and are mainly composed of biocompatible and biodegradable lipids that self-assemble in aqueous medium to form lipid bilayer membranes surrounding a hydrophilic core. Transfersomes were first introduced by Cevc et al. in which they added an edge activator (EA) or surfactant to the lipid components of liposomes [6]. The elasticity induced by the presence of surfactant allows the resulting transfersomes to become more flexible than standard liposomes, enhancing their ability to pass through small pores and minimising the possibility of bilayer rupture that could occur with rigid liposomes [7, 8]. Transfersomes have gained interest since they can be useful for both small molecular weight drugs and macromolecules, and may be delivered by many routes such as parenteral, transdermal or buccal [3, 9, 10].

Local anaesthetics (LA) such as lidocaine, bupivacaine, and ropivacaine block the transmission of painful stimuli to the brain by acting on ion channels of nociceptor fibres, to achieve a control for both acute and chronic pain [11]. In order to improve their pharmacokinetic properties, prolong their pharmacological activity and minimise their toxicity, many studies have formulated LAs in different forms such as injectable implants, films, microstructural systems, polymeric nanoparticles and lipid based vesicles [12, 13]. However, most of the available LA forms have short duration of action, and frequent application are required to get long term pain relief which inversely affect the patient compliance and increase the side effects. A sustained release formulation of LA would be a great option to overcome the adverse effects of previously reported formulations. Having sustained release LA would reduce the need for multiple administrations regardless the route of administration [14, 15]. Additionally, it would reduce the risks related to high dose applications. Moreover, sustained release LA formulation would help not only in producing anaesthesia for acute cases but would also aid the management of chronic pain.

There are few licensed LA solution formulations to produce a prolonged effect allowing for a decreased frequency of injection. The prolonged effect is mainly gained by either the addition of a vasoconstrictor e.g. epinephrine, which prevents the leakage of the LA to the blood stream, or through liposomal and lipid-based Depofoam formulations [16, 17]. To our knowledge, several attempts have been made to formulate LAs as polymeric or lipid based microspheres and nanoparticles for injectable or transdermal drug delivery. However, most of the systems developed were not intended for sustained release administration. Hence, the aim of this work is to formulate sustained release LA-loaded transfersomes without the need to add vasoconstrictor or to use the complicated formulation method of Depofoam.

Novel transfersomes loaded with LA such as lidocaine and intended to be a sustained release delivery system could be a better approach to improve patient compliance and achieve the required level of anaesthesia. However, the properties of drug loaded transfersomes usually vary with several parameters such as the nature of lipid and EA component, the concentration of each component, and the preparation process parameters [18]. Although previous studies have attempted to find the optimum composition of transfersomes with some desired properties [19, 20], a new optimisation study was required since both the drug and sustained release transfersomes have not been reported in the literature to date. Optimisation of properties such as size and entrapment efficiency were essential in order to obtain transfersomes that could produce the required level of anaesthesia without any systemic side effects. Therefore, this study aimed to optimise the composition of transfersome with respect to parameters such as the type of lipids and the EA employed, using a Taguchi design of experiments (DOE) and lidocaine free-base was chosen as a model LA drug.

Thus, the current study optimised the formulation parameters of a novel transfersome for sustained release of lidocaine by screening several types of lipid (natural and synthetic), several EAs (surfactants) with different HLB values, and several ratios of lipids to surfactants. Commonly, the more water-soluble lidocaine hydrochloride salt is used for injectable forms, but the free-base is preferable since it exhibits more lipophilic properties. However, in order to use the free-base lidocaine a novel analytical method using high performance liquid chromatography (HPLC) was also developed and validated according to the International Council for Harmonisation (ICH) guidelines [21] as a reliable and more robust method for quantification of lidocaine in comparison with usual UV spectrometry method.

2. Materials and methods

2.1 Materials

Egg phosphatidylcholine (EPC), sodium deoxycholate (SDC), Tween 80, Span 80, and phosphate buffered saline (PBS, pH7.4) tablets were purchased from Sigma Aldrich, UK. Lidocaine (free-base), acetonitrile (ACN), methanol (MeOH), phosphotungstic acid and absolute ethanol were obtained from Fisher Scientific, UK. Di-Potassium hydrogen orthophosphate anhydrous was purchased from BDH Chemicals Ltd, UK. 1,2-Dimyristoyl-sn-glycero-3-phosphorylcholine (DMPC) was purchased from Lipoid, Switzerland. All solvents used were of HPLC grade. Whatman® polycarbonate filter membranes of 25mm diameter and 0.2µm pore size were purchased from Sigma Aldrich, UK. Spectra/Pro®3 dialysis membrane tubing of mwco 3.5kD with diameter of 11.5mm was purchased from Fisher Scientific, UK.

2.2 Methods

2.2.1 HPLC chromatographic system

An HPLC system (1200 series) from Agilent Technologies, UK was used with diode-array detector (DAD) and Variable Wavelength Detector (VWD). An Agilent C18 column with dimensions of 4.6x150mm, and a particle size of 5µm was employed (Agilent Technologies, USA). The column oven temperature was set at 30 °C with an injection volume of 10µl. The final composition of the mobile phase was optimised with ACN and 0.01M phosphate buffer (70:30, v/v) at a flow rate of 1ml/min (the total run time was 5 minutes), and a UV detection wavelength of 255nm.

2.2.2 Method development and validation

To optimise the chromatographic conditions, the effect of several factors was analysed and investigated. These included mobile phase composition, flow rate, and detection wavelength (Table 1). The optimised method was then validated according to ICH-guidelines [21]. A stock solution of lidocaine and a set of diluted standard solutions were prepared. A calibration curve was plotted over the concentration range of 0.1 - 2mg/ml. Linearity was evaluated according to the regression value (R^2). Precision was confirmed by proving both repeatability and intermediate precision. Repeatability was assessed using a triplicate sample of 3 different lidocaine concentrations, while the intermediate precision was evaluated over 3 different days. The accuracy was measured by calculating the percent recovery and according to ICH by proving that the method is precise and linear. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were assessed according to equations 1 and 2 respectively.

Table 1. Parameters used in HPLC method development of lidocaine.

$$LOD = \frac{3.3 \times SD}{S} \qquad (1)$$

$$LOQ = \frac{10 \times SD}{S} \qquad (2)$$

Where SD is the standard deviation of the response, and S is the slope of the calibration line.

2.2.3 Taguchi DOE

Taguchi design was employed as a tool to optimise the transfersome formulation parameters. It was used to evaluate the effect of several formulation parameters such as the nature of lipid used (natural or synthetic), the type of surfactant (EA), and the ratio of lipid to surfactant (Table 2). The design was constructed using mixed level design (L18 array) with 3 factors, one factor has 2 levels and the other two factors has 3 levels (Table 2). Minitab® 18.1 software was used to construct the study design, and the selected response variables were investigated. The studied variables were transfersome size and entrapment efficiency (%EE). Table 3 summarises the composition of Taguchi design transfersome formulations. This design enables identifying and ranking the significant formulation factors that would have an effect on size and %EE.

Table 2. Taguchi design of experiment including various factors and levels.

Table 3. Summary of the composition of each formulation with different types of lipid (SPC, DMPC), EA (Span 80, Tween 80 and SDC) and their ratios (95:5, 75:25 and 55:45 w/w) with each other.

2.2.4 Preparation of transfersomes

Lidocaine-loaded transfersomes were prepared using a lipid-based film hydration method [9, 19, 22] (Figure 1). Transfersome formulations were prepared using compositions according to Taguchi DOE (Table 3). Lidocaine (70.29mg) was used in each formulation as the equivalent molar ratio to both phospholipid and EA ratio, and all components were dissolved in 12ml of absolute ethanol. The mixture was sonicated for 5 minutes to ensure complete dissolution of all components using a water bath sonicator (Model U500D, Ultrawave, UK). This was followed by the evaporation of ethanol using a rotary evaporator (Heidolph Laborota 4000 efficient, Germany) at 60°C under reduced pressure. A continuous thin lipid film was obtained upon rotation (at 250 rpm) and after the complete evaporation of the ethanol. The lipid film was hydrated using 9ml of PBS (pH 7.4). The hydrated formulation was left to anneal for 30 minutes. Subsequently, formed transfersomes were extruded (Liposofast LF 50, Avestin, Germany), for 5 cycles at 45°C using 200 nm membrane filters.

Figure 1. Schematic diagram of transfersomes preparation method.

2.2.5 Size, Polydispersity index (PDI), and Zeta potential

Transfersomes were characterised for particle size, polydispersity index, and zeta potential using a dynamic light scattering (DLS) instrument (Zetasizer Nano; Malvern Instruments Ltd., UK). In this

respect, 1ml of each formulation was transferred into the transparent Malvern zeta potential cuvette and placed in the instrument.

2.2.6 Entrapment efficiency (%EE)

The total drug concentration contained within each formulation was measured using a 0.5ml aliqout, which was diluted with methanol until a clear transparent solution was obtained. The drug concentration was then measured using the developed HPLC method (section 2.2.1). Another 0.5ml of the formulation was placed in a centrifugal filter tube of 3kDa pore size (Amicon® Ultra, Merck Millipore Ltd, Ireland) and centrifuged for 30 minutes with centrifugal force of 15.6 rcf using benchtop centrifuge (Eppendorf Centrifuge 5415 D, Germany). The filtrate containing free drug at the bottom was diluted with methanol, and the concentration was measured via HPLC. The %EE was then calculated using equation 3.

$$\%EE = \frac{(total\ drug\ conc. - unentrapped\ drug\ conc.)}{Total\ drug\ conc.} \times 100 \qquad (3)$$

2.2.7 Transfersome morphology

Transfersome morphology was observed under a transmission electron microscope (TEM) using a FEI Morgagni Transmission Electron Microscope (Philips Electron Optics BV, Netherlands). A drop of the transfersome suspension was placed on the copper grid and left for few minutes. A drop of negative stain solution (phosphotungstic acid (1%)) was then added to the sample grid. The grid was then rinsed with distilled water to wash off the excess stain, and placed in the TEM sample chamber for visualisation.

2.2.8 In-vitro release study

The release profiles of selected samples based on DOE results were studied in vitro at 37˚C in addition to a control sample containing free lidocaine only. The release study was carried out in 500m vessels filled with 150ml PBS (pH7.4) on a magnetic stirrer at speed of 250rpm. Transfersome samples were sealed inside dialysis cellulose membrane with 3.5kD molecular exclusion pores, measuring 12cm, which were suspended in the PBS medium and incubated at 37˚C. Aliquots of 0.5ml of each sample were withdrawn at time intervals 0, 1, 3, 5, 7, 16 and 24 h. and replaced with a fresh PBS solution. The aliquots were then analysed using the developed HPLC method for the lidocaine content.

2.2.9 Statistical analysis

All data are presented as means with standard deviation (SD). A statistical analysis was performed on the data using one-way ANOVA with Tukey post-test or unpaired *t*-test analysis employing Minitab® 18.1 software to determine any significant differences between the studied variables. The level of significance in difference was considered as $p<0.05$.

3. Results and discussion

3.1 HPLC method development

Development of an HPLC method as an accurate method for the analysis of lidocaine free-base was necessary because the UV spectrophotometer was the most reported way to quantify the free-base, and all other reported HPLC methods were developed for the analysis of lidocaine HCl [23-27]. Several methods suggested the use of ACN and PBS as mobile phase components for lidocaine analysis [23-26]. To achieve good peak resolution, the use of a mobile phase with a basic pH has been suggested, while others have proposed the use of MeOH instead of ACN [27]. Therefore, method optimisation and validation were required in order to improve lidocaine free-base analysis and investigation in formulations. The mobile phase composition, PBS molarity, detection wavelength and flow rate were adjusted to achieve high chromatographic resolution, and short retention time (Rt) of lidocaine free-

base. Twenty methods were designed to cover the variables and DAD was used to scan the absorbance over a range of wavelengths (Table 4). Upon applying these methods, it was found that some led to chromatograms showing poor peak shapes including broad peaks, fronting and tailing peaks (Table 4). Additionally, some methods resulted in a long Rt of lidocaine such as M7, M14 and M16 (11.30 min, 14.30 min and 20.44 min, respectively). Further detection with methods employing slower flow rates (M8, M9, M15, M17, and M18) was not carried out, since a short run time was the aim of the method development. Among all methods, only three methods (M10, M11, and M12) gave a sharp peak with symmetry more than 0.90 and short retention time of lidocaine. However, M10 with 1.5ml/min flow rate was excluded as the drug peak occurred immediately after the solvent front. Method M11, employing a mobile phase of 30% PBS (0.01 M) and 70% ACN (%v/v), a flow rate 1 ml/min and a detection wavelength of 255 nm was chosen as it showed the highest peak resolution (Figure 2), and absorbance, as well as a reasonable test run time (5 minutes in total).

Table 4. List of methods tested in the HPLC method development study including different mobile phase composition, flow rate and detection wavelengths, with the obtained results including their Rt, symmetry, and shape.

Figure 2. Representative lidocaine HPLC chromatogram obtained using method M11.

3.2 HPLC method validation

The validation parameters of the developed method are shown in Tables 5 and 6. The data showed good compliance with ICH guidelines [21]. The analytical method revealed a linear relationship over the concentration range studied and the method was proven to be accurate and precise.

Table 5. Validation parameters of lidocaine free-base.

Table 6. Accuracy and precision (intermediate precision and repeatability).

3.3 Transfersome preparation and characterisation

3.3.1 Transfersome optimisation and Taguchi DOE analysis

Taguchi design was used to determine the best formulation combination to achieve transfersomes with the minimum size and the highest %EE. The choice of design parameters was determined by previously reported studies. The type of phospholipids used has been investigated in several studies and has been reported to produce liposomal vesicles with different properties [28]. Therefore, natural and synthetic phospholipids (EPC and DMPC) were investigated in this study. Additionally, it was reported that the surfactant type and concentration can also affect transfersome size and %EE [18]. For this reason, three surfactants (EA) were used in this DOE in three different percentages (Table 2). Lidocaine-loaded transfersomes were prepared according to the designed experiments (Table 3). Transfersomes were then characterised as mentioned in the methods section for size, charge, morphology, and %EE (Table 7).

Table 7. Transfersome formulation (F1-F18) characterisation results; size, polydispersity index (PDI), zeta potential, and entrapment efficiency (%EE).

Transfersomes obtained by running the 18 experiments showed sizes below 208.70 ± 4.66 nm with consistent PDI, except two samples (F3 and F18), where transfersome sizes were unexpectedly large (515.35 ± 4.45nm and 671 ± 15.13nm respectively). Larger transfersomes may have been formed in these cases due to the high level of surfactant that was used, as it was previously reported that increased liposome size resulted from the presence of surfactant molecules situated within the lipid bilayer in a way that increased its diameter [29]. Additionally, the %EE results obtained from all 18 formulations ranged from 44.26 ± 2.61% (F 1) to 56.97 ± 0.18% (F 9). Statistical analysis of the obtained data was performed. The %EE results of all 18 formulations showed no significant differences (P> 0.05). The low encapsulation efficiency was suggested to be related to the size of transfersome. As the drug has lipophilic properties it should be entrapped within the lipid bilayer, however, the small size does not offer enough space within the bilayer for large amount of drug to accommodate. Higher entrapment would be expected with micron-sized transfersomes [19], however, nanosized transfersomes are preferable for any route of delivery, including buccal [30, 31], transdermal [32, 33] and parenteral [34]. It is claimed that both the nanosize and the deformability of transfersomes are the key characteristics for enhanced tissue permeation when administered topically (transdermal or buccal), in addition to the improved solubility, targeting and stability when injected intravenously [32, 34, 35].

In contrast, statistical analysis of transfersome size showed a considerable variation (Figure 3). There was a significant difference in size between most samples that included 5% and 25% EA in comparison to 45%, with *P* values of either **P< 0.05 or ***P<0.001* (Figure 3 A, B C, E and F). A small but significant difference (with *P<0.05*) was also noted between samples that included EA of 5% in comparison to samples that had 25% EA (Figure 3 A, B, E, and F).

The results were analysed using Taguchi analysis in Minitab® 18 software, in which the variation of the response was studied using signal to noise (S/N) ratio. S/N ratio is a quality analytical parameter which is useful to determine the best level of each experimental parameter. The type of S/N ratio is selected depending on the desired characteristic. A larger S/N ratio was considered desirable for the analysis of the %EE results, as a high level of drug entrapment is desirable. However, because there was not a significant difference between the %EE results of the 18 formulations, the Taguchi analysis was carried out considering only transfersome size. In this case, a small S/N ratio was considered desirable for the analysis, as smaller transfersome size is preferable (Table 8). The analysis enabled ranking the studied parameters in accordance with the magnitude of their effect on the size of transfersomes. Both the type of surfactant and its concentration were clearly observed to be the most significant factors affecting transfersome sizes (Table 8). Additionally, samples that were prepared with Tween 80 showed the smallest size, followed by SDC and Span 80 (Figure 4). Furthermore, it was also found that the employed concentration of the EA has a noticeable effect on transfersome size, since increasing the concentration from 5% to 25% clearly resulted in a size reduction. However, further increment of surfactant concentration up to 45% showed a contrasting effect (Figure 4). Transfersome sizes were increased in formulations with a high surfactant concentration as a result of the molecular repulsion that possibly occurred between the surfactant and phospholipid molecules within transfersome bilayers [18, 29]. Additionally, the analysis also showed that the use of either natural or synthetic phospholipids had no significant effect on the size of transfersomes and their ability to entrap drug.

Figure 3. Graphical representation of transfersomes size of all 18 formulations.

Table 8. Response table for Signal to Noise ratios (S/N), showing the rank of the factors X1, X2 and X3 as they affect transfersome size.

Figure 4. Effect of studied parameters using the mean of S/N ratios.

3.3.2 Zeta potential

Measuring zeta potential of the obtained transfersomes was crucial to determine since it was claimed that it could affect the permeation rate when they administered transdermally or through the buccal tissue [20]. For example it was reported that transfersome with high negative charge will effectively enhance their skin permeation [7]. The results revealed that all prepared transfersomes hold a negative charge (Table 7). This can be explained as the net surface charge of the transfersomes depends mainly on both phospholipid and surfactant character. Both EPC and DMPC are zwitterionic compounds with an isoelectric point of 6-7, meaning that at the current experimental conditions (pH = 7.4) they hold a net negative charge [20, 36]. Moreover, samples that were prepared using SDC (F7, F8, F9, F16, F17, and F18) were observed to carry a higher negative charge than Tween 80 and Span 80 based samples. Additionally, the negative charge increased dramatically from -6.86 mV up to -21.70 mV as the SDC concentration increased from 5% (formulation F7) to 45% (F9). Similarly, it increased from -3.60mV to -18.65 mV in formulations F16 to F18, which was in good agreement with the literature since the surfactant concentration affects the final charge of the lipid vesicles [20, 37].

3.3.3 Transfersome morphology

The morphology of lidocaine loaded transfersomes was observed using TEM (Figure 5). Transfersomes displayed a uniform, spherical shape with intact bilayer membrane, and unilamellar bilayer structure. TEM images confirmed that transfersomes were effectively achieved and they were found to be approximately 200nm in size.

Figure 5. TEM images of transfersomes produced by F4.

3.3.4 In-vitro release

According to the optimisation study results, 6 samples were selected for the in vitro-release study. As the Lipid: EA ratio had the major effect on the transfersomes properties, the samples were chosen from the ratio that gave the desired properties, which was 75:25 of lipid: EA (Table 8). Although the lipid type did not show a significant effect (p>0.05) from the optimisation results, the selected six samples were prepared using both lipids; EPC (F2, F5, and F8) and DMPC (F11, F14, and F17) in order to check their effect on the release profile. However, as the EA type was the second important factor to produce the desired transfersomes all 3 EA were considered in the selection of samples (Table 7). The release profile of the 6 samples in addition to a control of free drug was studied over 24 hours. The cumulative amount of drug released was calculated for each formulation (Table 9). The release profile of the control sample (free drug) revealed that 96% drug amount passed across the dialysis membrane by 1 hour, while all 6 transfersome samples showed complete drug release at 24h (Table 9, Figure 6). Transfersomes samples that were prepared by EPC not only showed sustained release of lidocaine but also a delayed release, with < 2% drug released after 1h (F2, F5, and F8), but subsequently almost 60% after 3h (Table 9). DMPC-based transfersomes showed between 5-21% drug release after 1h. The release profiles of the six formulations proved that transfersomes were successfully optimised and prepared to sustain the release lidocaine over 24h. Moreover, it is a

promising system to deliver LA via any route with a reduced frequency of administration, which in turn would reduce side effects and enhance the pain management (both acute and chronic) [15, 17].

Table 9. In-vitro release study results, % drug released over several time points, n=3 ± SD

Figure 6. Release profile of lidocaine permeated across dialysis bag from 6 different transfersomes formulations versus the free drug (control) over 24h.

Conclusion

In this study, transfersomes were optimised to develop sustained release delivery system of lidocaine. They were formulated using a simple lipid film hydration method. Preparation parameters were optimised using a Taguchi DOE in terms of phospholipid type, type of EA and ratio of phospholipid to EA. Transfersomes were characterised for size, PDI, charge, and %EE. The obtained transfersomes were approximately 200 nm in size with PDI ≤ 0.3. To determine the entrapment efficiency, a new HPLC method for lidocaine was optimised and validated according to ICH guidelines. The proposed method was validated for linearity, accuracy, sensitivity, intermediate precision and repeatability, and was shown to be valid to for the analysis of lidocaine free-base according to ICH guidelines. The calculated %EE varied as the formulation parameters changed, but was generally between 44-56%. Analysing the data obtained by Taguchi DOE showed that the effect of formulation factors on both size and %EE were in the following rank order: lipid: EA ratio >EA type >lipid type. The type of lipid (natural or synthetic) showed no significant effect on transfersome size. Increasing the EA concentration up to 25% resulted in a reduction in transfersomes size; however, with a further increase in EA, transfersome size was seen to increase.

Transfersome samples were selected based on the analysis of the optimisation results, and their release profiles were assessed. All 6 samples proved that the optimised transfersomes can be used as a sustained release delivery system of LA as they released lidocaine slowly over 24h in contrast to the free drug that showed complete drug release by 1 hour.

These samples will be employed in future work for testing their ex-vivo release and permeability profile.

Table 7. Parameters used in HPLC method development of lidocaine.

Parameters	Value
Mobile phase solution	- 0.01M Phosphate buffer: Acetonitrile (70:30, 50:50, 30:70) - Water: Methanol (30:70,50:50, 70:30) - 0.1M Phosphate buffer: Acetonitrile (30:70)
Detection wavelength	220 - 290 nm
Flow rate	0.5 ml/min, 1 ml/min, 1.5 ml/min

Table 8. Taguchi design of experiment including various factors and levels.

Factors (X)	Level		
	1	2	3
Lipid type (X1)	EPC (natural lipid)	DMPC (synthetic lipid)	-
EA (HLB) (X2)	Span 80 (4.3)	Tween 80 (15)	SDC (16)
Lipid:EA (X3)	95:5	75:25	55:45

Table 9. Summary of the composition of each formulation with different types of lipid (SPC, DMPC), EA (Span 80, Tween 80 and SDC) and their ratios (95:5, 75:25 and 55:45 w/w) with each other.

Formulation	X1 (lipid type)	X2 (EA)	X3 (Lipid:EA)
F1	EPC	Span 80	95:5
F2	EPC	Span 80	75:25
F3	EPC	Span 80	55:45
F4	EPC	Tween 80	95:5
F5	EPC	Tween 80	75:5
F6	EPC	Tween 80	55:45
F7	EPC	SDC	95:5
F8	EPC	SDC	75:5
F9	EPC	SDC	55:45
F10	DMPC	Span 80	95:5
F11	DMPC	Span 80	75:5
F12	DMPC	Span 80	55:45
F13	DMPC	Tween 80	95:5
F14	DMPC	Tween 80	75:5
F15	DMPC	Tween 80	55:45
F16	DMPC	SDC	95:5
F17	DMPC	SDC	75:5
F18	DMPC	SDC	55:45

Table 10. List of methods tested in the HPLC method development study including different mobile phase composition, flow rate and detection wavelengths, with the obtained results including their Rt, symmetry, and shape.

Method	Mobile phase composition (% v/v)	Flow rate (ml/min)	Rt (minutes)	Peak symmetry	Peak shape
M1	Water: MeOH (30:70)	1.5	4.83	0.71	broad and tailing peak
M2	Water: MeOH (30:70)	1	7.23	0.60	broad and tailing peak
M3	Water: MeOH (30:70)	0.5	8.08	0.67	sharp peak
M4	Water: MeOH (50:50)	1.5	8.60	0.43	broad and tailing peak
M5	Water: MeOH (50:50)	1	5.38	0.44	broad and tailing peak
M6	Water: MeOH (50:50)	0.5	3.08	0.58	fronting peak
M7	Water: MeOH (70:30)	1.5	11.30	0.44	broad and tailing peak
M8	Water: MeOH (70:30)	1	-	-	-
M9	Water: MeOH (70:30)	0.5	-	-	-
M10	**PBS (0.01M): ACN (30:70)**	**1.5**	**1.59**	**0.90**	**sharp peak**
M11	**PBS (0.01M): ACN (30:70)**	**1**	**2.84**	**0.93**	**sharp peak**
M12	**PBS (0.01M): ACN (30:70)**	**0.5**	**4.46**	**0.95**	**sharp peak**
M13	PBS (0.01M): ACN (50:50)	1.5	3.66	0.71	fronting peak
M14	PBS (0.01M): ACN (50:50)	1	14.30	0.73	fronting peak
M15	PBS (0.01M): ACN (50:50)	0.5	-	-	-
M16	PBS (0.01M): ACN (70:30)	1.5	20.44	0.58	tailing peak
M17	PBS (0.01M): ACN (70:30)	1	-		-
M18	PBS (0.01M): ACN (70:30)	0.5	-		-
M19	PBS (0.1M): ACN (30:70)	1.5	1.59	0.91	fronting peak
M20	PBS (0.1M): ACN (30:70)	1	2.49	0.79	fronting peak

Table 11. Validation parameters of lidocaine free-base.

Parameter	Value
(y= ax ± b)	y= 1106.2x-0.1751
R^2	0.9999
Linearity range	0.1-2 mg/ml
LOD	$1.55x10^{-07}$ mg/ml
LOQ	$4.72x10^{-07}$ mg/ml

Table 12. Accuracy and precision (intermediate precision and repeatability).

Initial Concentration (mg/ml)	Day 1		Day 2		Day 3	
	Accuracy (%)	*RSD (%)*	*Accuracy (%)*	*RSD (%)*	*Accuracy (%)*	*RSD (%)*
0.2	100.43	0.08	97.60	0.06	97.70	0.09
0.4	99.56	0.05	97.95	0.01	96.93	0.09
0.7	100.95	0.10	98.52	0.12	98.48	0.10

Table 7. Transfersome formulation (F1-F18) characterisation results; size, polydispersity index (PDI), zeta potential, and entrapment efficiency (%EE).

Formulation	Size (nm)	PDI	Zeta potential (mV)	EE (%)
F1	188.60 ± 9.33	0.18 ± 0.02	-4.66 ± 1.42	44.26 ± 2.61
F2	208.70 ± 4.66	0.25 ± 0.03	-4.56 ± 0.29	51.73 ± 1.51
F3	515.35 ± 4.45	0.29 ± 0.12	-7.49 ±0.84	53.87 ± 5.73
F4	181.05 ± 11.66	0.18 ± 0.03	-3.14 ± 0.21	45.82 ± 3.98
F5	146.95 ± 0.63	0.22 ± 0.02	-2.15 ± 1.20	49.83 ± 2.07
F6	121.15 ± 1.34	0.22 ± 0.04	-2.77 ± 0.05	50.19 ± 2.02
F7	195.95 ± 7.28	0.14 ± 0.00	-6.86 ± 1.16	53.05 ± 0.62
F8	98.93 ± 2.07	0.14 ± 0.01	-17.10 ± 0.28	54.55 ± 1.05
F9	134.85 ± 12.51	0.26 ± 0.03	-21.70 ± 0.28	56.97 ± 0.18
F10	188.00 ± 22.06	0.13 ± 0.00	-1.28± 1.33	50.76 ± 0.72
F11	200.35 ± 19.02	0.21 ± 0.01	-3.25 ± 0.34	48.93 ± 4.12
F12	194.30 ± 15.41	0.15 ± 0.00	-4.65 ± 0.03	53.07 ± 4.44
F13	171.15 ± 3.18	0.19 ± 0.00	-0.48 ± 0.76	52.96 ± 4.44
F14	96.92 ± 16.37	0.17 ± 0.01	-1.15 ± 1.17	50.67 ± 0.21
F15	116.00 ± 6.64	0.19 ± 0.01	3.94 ± 2.12	50.80 ± 1.22
F16	153.70 ± 28.28	0.15 ± 0.01	-3.60 ± 0.57	50.93 ± 2.07
F17	146.05 ± 0.35	0.30 ± 0.02	-13.20 ± 1.27	52.63 ± 0.12
F18	671.00 ± 15.13	0.59 ± 0.16	-18.65 ± 1.62	55.72 ± 7.33

Table 8. Response table for Signal to Noise ratios (S/N), showing the rank of the factors X1, X2 and X3 as they affect transfersome size.

Level	X1	X2	X3
1	-45.03	-47.24	-45.07
2	-45.52	-42.74	-43.12
3		-45.84	-47.64
Delta	0.49	4.50	4.52
Rank	3	2	1

Table 9. In-vitro release study results, % drug released over several time points, n=3 ± SD

Time points	Formulation codes (% release ± SD)						
	EPC			DMPC			Control
	F2	F5	F8	F11	F14	F17	
1h	0	1.37 ± 2.37	0	5.55 ± 0.34	21.35 ± 0.83	16.29 ± 11.40	96.20 ± 3.23
3h	59.76 ± 14.82	56.99 ± 6.37	62.08 ± 6.26	85.94 ± 3.01	70.65 ± 2.70	47.25 ± 0.24	98.53 ± 6.35
5h	89.47 ± 7.87	78.25 ± 1.66	91.68 ± 1.44	87.27 ± 4.82	86.01 ± 5.14	70.88 ± 3.17	
7h	93.86 ± 6.38	84.60 ± 0.98	96.13 ± 2.43	91.01 ± 2.89	92.33 ± 5.93	94.51 ± 3.90	
16h	96.63 ± 6.85	90.56 ± 1.23	98.64 ± 1.56	92.98 ± 3.23	96.35 ± 6.32	97.68 ± 4.21	
24h	98.90 ± 7.84	93.96 ± 3.15	100.00 ± 1.69	95.61 ± 6.49	101.24 ± 14.33	100.36 ± 5.97	

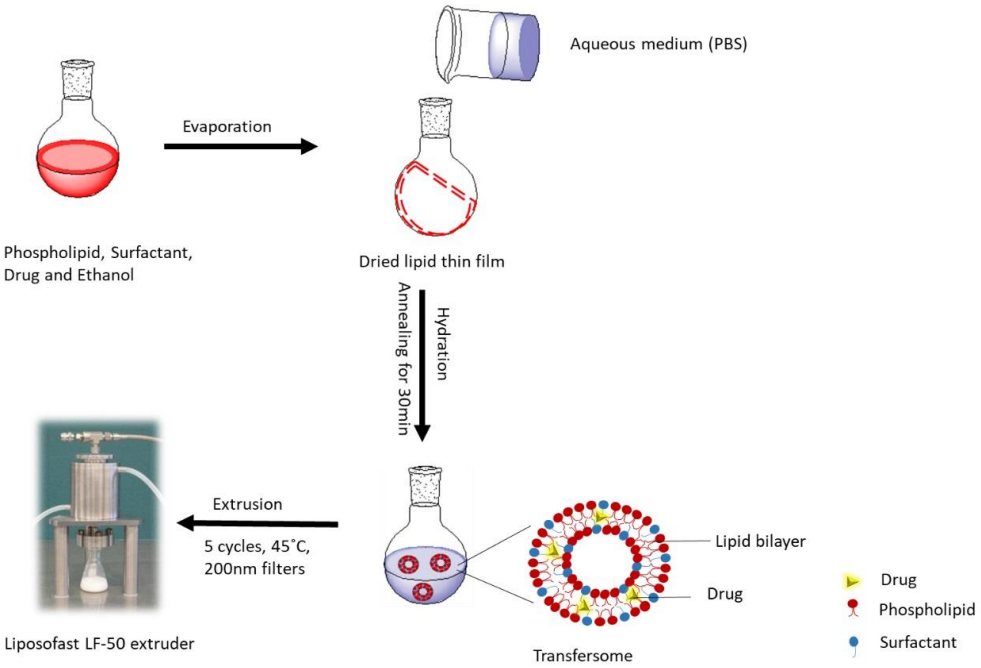

Figure 3. Schematic diagram of transfersomes preparation method.

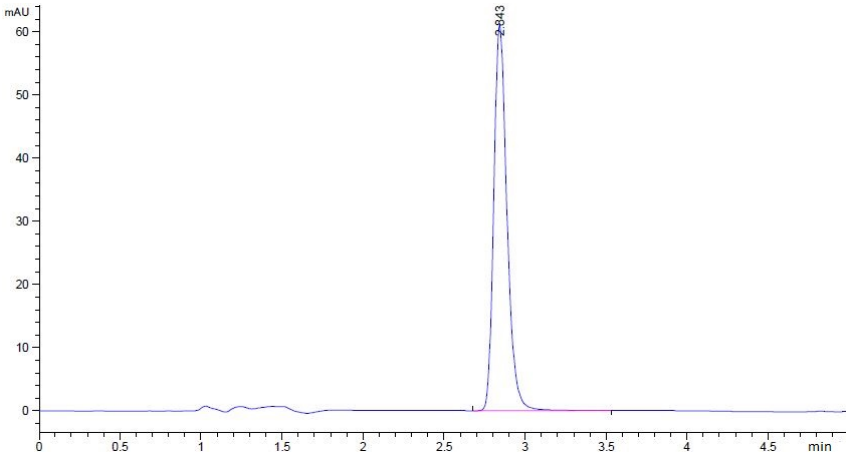

Figure 4. Representative lidocaine HPLC chromatogram obtained using method M11.

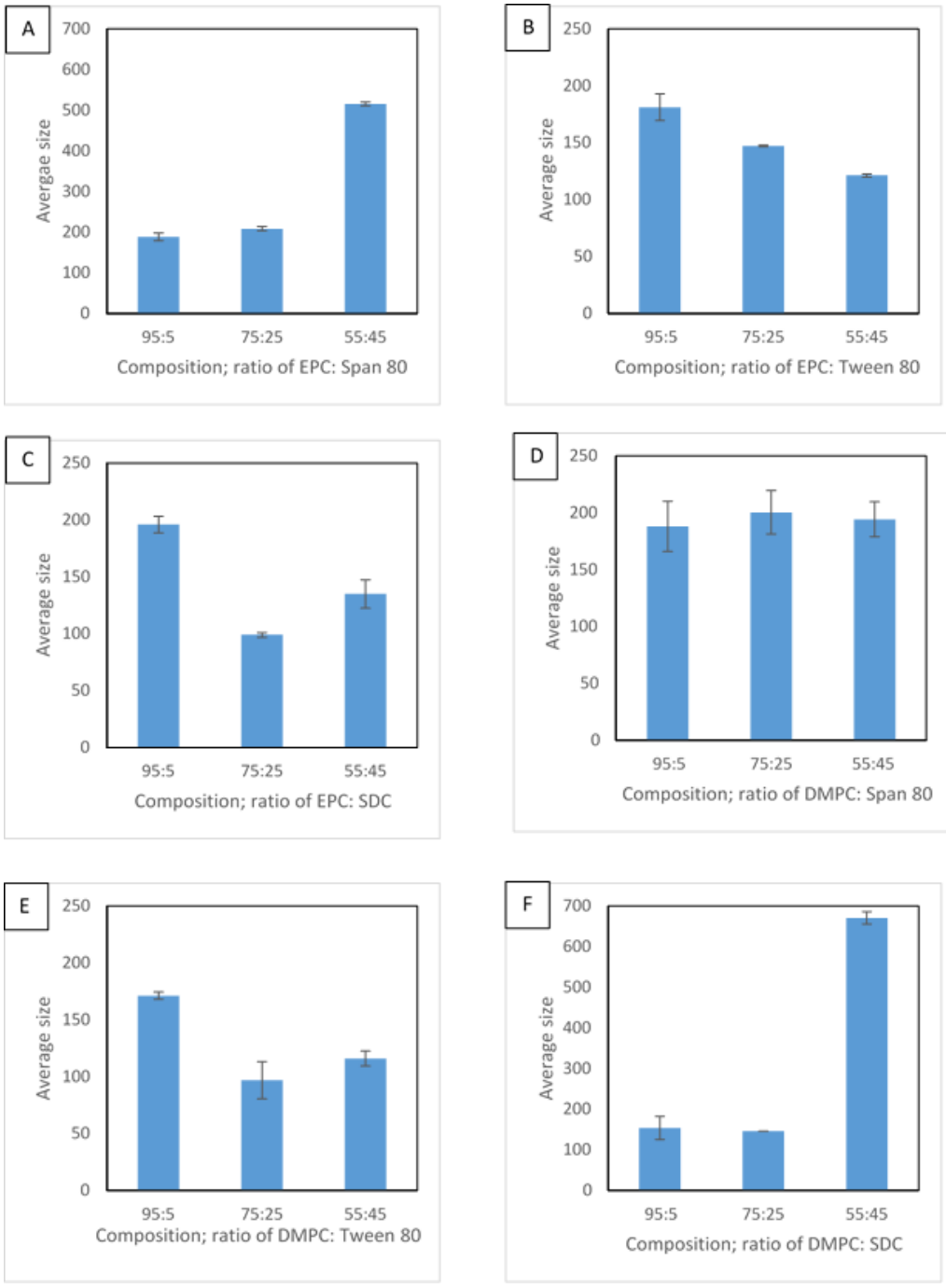

Figure 3. Graphical representation of transfersomes size of all 18 formulations.

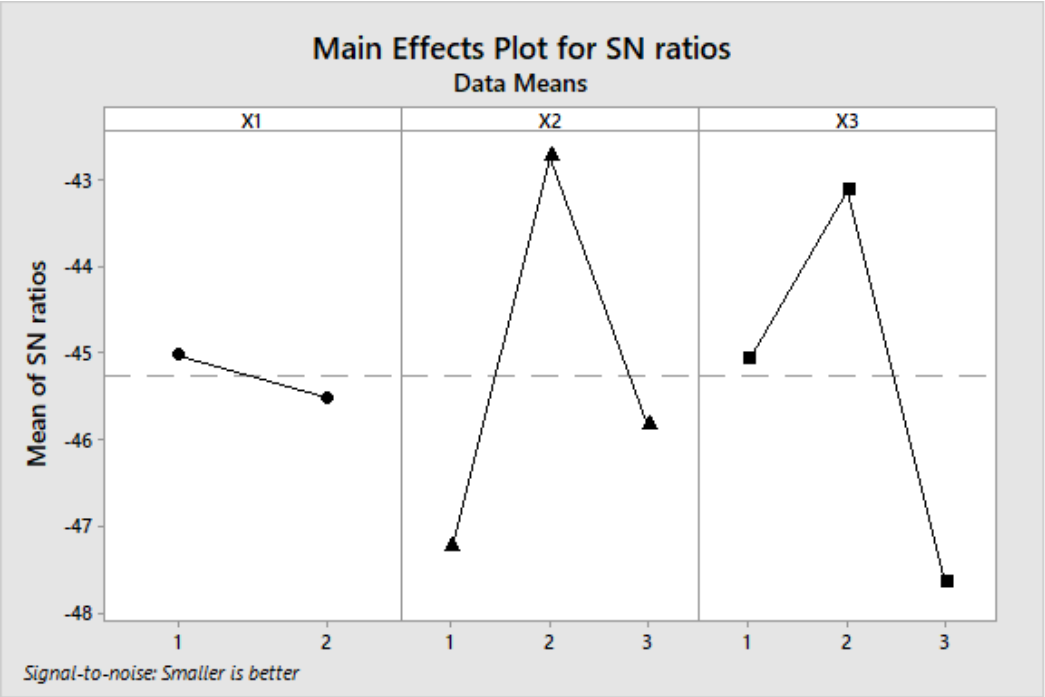

Figure 4. Effect of studied parameters using the mean of S/N ratios.

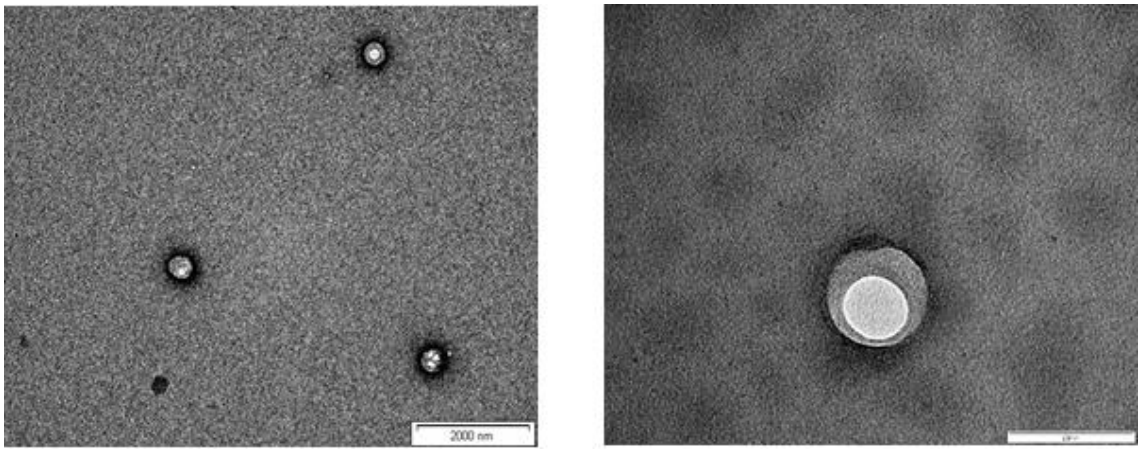

Figure 5. TEM images of transfersomes produced by F4.

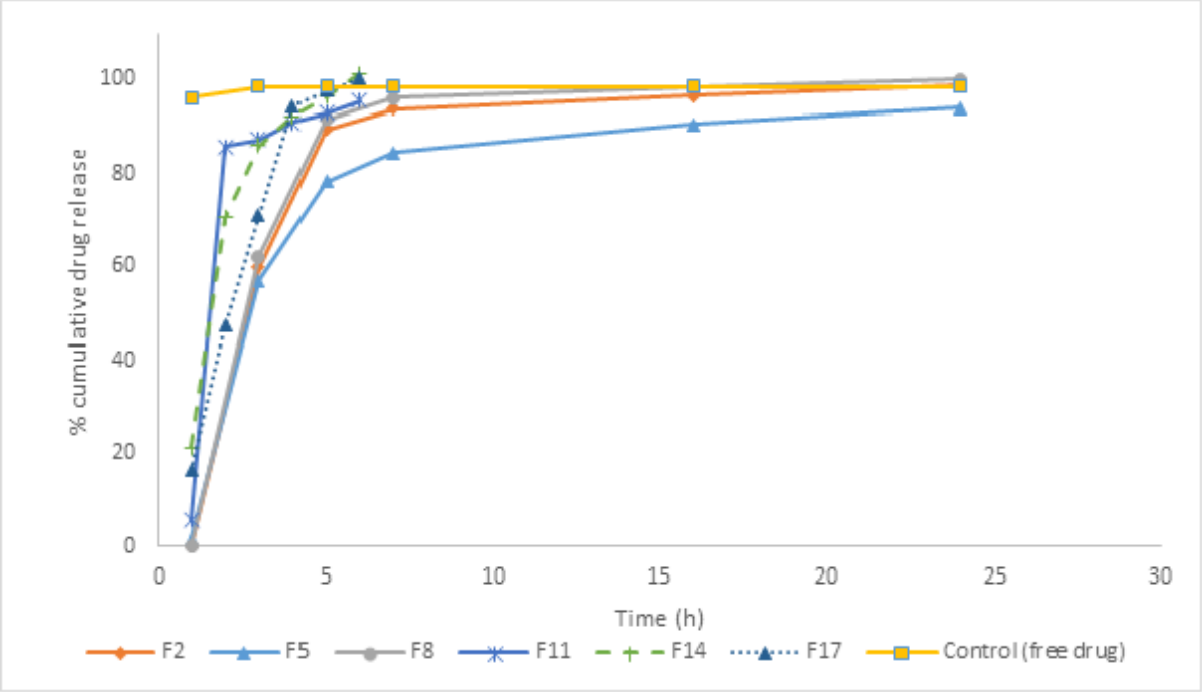

Figure 6. Release profile of lidocaine permeated across dialysis bag from 6 different transfersomes formulations versus the free drug (control) over 24h.

References

1. Biju SS, Talegaonkar S, Mishra PR, Khar RK. Vesicular systems: an overview. *Indian J Pharm Sci.* 2006;68(2).
2. Karim KM, Mandal AS, Biswas N, et al. Niosome: a future of targeted drug delivery systems. *J Adv Pharm Technol Res.* 2010;1(4): 374-380.
3. Ascenso A, Raposo S, Batista C, et al. Development, characterization, and skin delivery studies of related ultradeformable vesicles: transfersomes, ethosomes, and transethosomes. *Int J Nanomedicine.* 2015;10: 5837-5851.
4. Khan I, Yousaf S, Subramanian S, et al. Proliposome powders prepared using a slurry method for the generation of beclometasone dipropionate liposomes. *Int J Pharm.* 2015;496(2): 342-350.
5. Rattanapak T, Young K, Rades T, Hook S. Comparative study of liposomes, transfersomes, ethosomes and cubosomes for transcutaneous immunisation: characterisation and in vitro skin penetration. *Journal of Pharmacy and Pharmacology.* 2012;64(11): 1560-1569.
6. Seddon JM, Cevc G. *Lipid polymorphism: structure and stability of lyotropic mesophases of phospholipids*: Marcel Dekker, Inc: New York, NY, USA; 1993.
7. Malakar J, Sen SO, Nayak AK, Sen KK. Formulation, optimization and evaluation of transferosomal gel for transdermal insulin delivery. *Saudi Pharm J.* 2012;20(4): 355-363.
8. Ali MH, Kirby DJ, Mohammed AR, Perrie Y. Solubilisation of drugs within liposomal bilayers: alternatives to cholesterol as a membrane stabilising agent. *Journal of Pharmacy and Pharmacology.* 2010;62(11): 1646-1655.
9. Jain S, Jain P, Umamaheshwari RB, Jain NK. Transfersomes a novel vesicular carrier for enhanced transdermal delivery: development, characterization, and performance evaluation. *Drug Dev Ind Pharm.* 2003;29(9): 1013-1026.
10. Pawar AY, Jadhav KR, Chaudhari LH. Transfersome a novel technique which improves transdermal permeability. *Asian J Pharm.* 2016;10(4): 425 -436
11. Rogobete AF, Dragomirescu M, Bedreag OH, et al. New aspects of controlled release systems for local anaesthetics: A review. *Trends in Anaesthesia and Critical Care.* 2016;9: 27-34.
12. Chahar P, Cummings KC. Liposomal bupivacaine: a review of a new bupivacaine formulation. *Journal of Pain Research.* 2012;5: 257-264.
13. Zorzetto L, Brambilla P, Marcello E, et al. From micro- to nanostructured implantable device for local anesthetic delivery. *International Journal of Nanomedicine.* 2016;11: 2695-2709.
14. Balocco AL, Van Zundert PGE, Gan SS, Gan TJ, Hadzic A. Extended release bupivacaine formulations for postoperative analgesia: an update. *Current Opinion in Anesthesiology.* 2018;31(5): 636-642.
15. Natarajan JV, Nugraha C, Ng XW, Venkatraman S. Sustained-release from nanocarriers: a review. *Journal of Controlled Release.* 2014;193: 122-138.
16. Giovannitti Jr JA, Rosenberg MB, Phero JC. Pharmacology of Local Anesthetics Used in Oral Surgery. *Oral Maxillofac Surg Clin North Am.* 2013;25(3): 453-465.
17. Rogobete AF, Dragomirescu M, Bedreag OH, et al. New aspects of controlled release systems for local anaesthetics: A review. *Curr Anaesth Crit Care.* 2016;9: 27-34.
18. Bnyan R, Khan I, Ehtezazi T, et al. Surfactant Effects on Lipid-Based Vesicles Properties. *J Pharm Sci.* 2018.
19. Ahmed TA. Preparation of transfersomes encapsulating sildenafil aimed for transdermal drug delivery: Plackett-Burman design and characterization. *J Liposome Res* 2015;25(1): 1-10.
20. Shaji J, Lal M. Preparation, optimization and evaluation of transferosomal formulation for enhanced transdermal delivery of a COX-2 inhibitor. *Int J Pharm Pharm Sci.* 2014;6(1): 467-477.
21. Guideline IHT. Validation of analytical procedures: text and methodology Q2 (R1). *International Conference on Harmonization.* Geneva, Switzerland; 2005:11-12.
22. El Zaafarany GM, Awad GA, Holayel SM, Mortada ND. Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. *Int J Pharm.* 2010;397(1-2): 164-172.

23. Abdelwahab NS, Nouruddin W, Fatatry H, Osman W. Determination of Thiomersal, Lidocaine and Phenylepherine in their Ternary Mixture. *J Chromatogr Sep Tech.* 2013;4(8): 1-6.
24. Carafa M, Santucci E, Lucania G. Lidocaine-loaded non-ionic surfactant vesicles: characterization and in vitro permeation studies. *Int J Pharm.* 2002;231(1): 21-32.
25. Qin WW, Jiao Z, Zhong MK, et al. Simultaneous determination of procaine, lidocaine, ropivacaine, tetracaine and bupivacaine in human plasma by high-performance liquid chromatography. *J Chromatogr B.* 2010;878(15-16): 1185-1189.
26. Ricci Júnior E, Bentley MVLB, Marchetti JM. HPLC assay of lidocaine in in vitro dissolution test of the Poloxamer 407 gels. *Rev Farm Bioquim Univ Sao Paulo.* 2002;38: 107-111.
27. Shaalan RA, Belal TS. HPLC-DAD stability indicating determination of nitrofurazone and lidocaine hydrochloride in their combined topical dosage form. *J Chromatogr Sci.* 2010;48(8): 647-653.
28. Sakdiset P, Okada A, Todo H, Sugibayashi K. Selection of phospholipids to design liposome preparations with high skin penetration-enhancing effects. *J Drug Deliv Sci Technol.* 2018;44: 58-64.
29. Barbosa RM, Severino P, Prete PS, Santana MH. Influence of different surfactants on the physicochemical properties of elastic liposomes. *Pharm Dev Technol.* 2017;22(3): 360-369.
30. Shojaei AH. Buccal mucosa as a route for systemic drug delivery: a review. *J Pharm Pharm Sci.* 1998;1(1): 15-30.
31. Rossi S, Sandri G, Caramella CM. Buccal drug delivery: A challenge already won? *Drug Discov Today Technol.* 2005;2(1): 59-65.
32. Zeb A, Qureshi OS, Kim H-S, Cha J-H, Kim H-S, Kim J-K. Improved skin permeation of methotrexate via nanosized ultradeformable liposomes. *International journal of nanomedicine.* 2016;11: 3813-3824.
33. Bragagni M, Mennini N, Maestrelli F, Cirri M, Mura P. Comparative study of liposomes, transfersomes and ethosomes as carriers for improving topical delivery of celecoxib. *Drug Delivery.* 2012;19(7): 354-361.
34. Sarfraz M, Afzal A, Yang T, et al. Development of Dual Drug Loaded Nanosized Liposomal Formulation by A Reengineered Ethanolic Injection Method and Its Pre-Clinical Pharmacokinetic Studies. *Pharmaceutics.* 2018;10(3): 151.
35. Malam Y, Loizidou M, Seifalian AM. Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. *Trends in Pharmacological Sciences.* 2009;30(11): 592-599.
36. Friedman S, Kolusheva S, Volinsky R, Zeiri L, Schrader T, Jelinek R. Lipid/polydiacetylene films for colorimetric protein surface-charge analysis. *Anal Chem.* 2008;80(20): 7804-7811.
37. Al Shuwaili AH, Rasool BK, Abdulrasool AA. Optimization of elastic transfersomes formulations for transdermal delivery of pentoxifylline. *Eur J Pharm Biopharm.* 2016;102: 101-114.