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Nanoemulsion formulation and characterization techniques: A review study

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Abstract :
Nanoemulsions are colloidal dispersion systems that are thermodynamically stable, composed of two immiscible liquids mixed along with emulsifying agents (surfactants and co-surfactants) to form a single phase. Nanoemulsions have extensively been investigated as drug delivery systems. This review aims to provide consolidated information regarding various formulation and characterization techniques developed for nanoemulsions. Nanoemulsions are formulated using two different methods, the persuasion method and the Brute force method. Various characterization techniques for nanoemulsions include determination of entrapment efficiency, particle size, polydispersity index, zeta potential as well as characterization through differential scanning calorimetry, Fourier-transform infrared spectroscopy and transmission electron microscopy. Nanoemulsions are further evaluated by studying in vitro drug release, in vitro permeation, stability and thermodynamic stability, shelf life, dispersibility, viscosity, surface tension, friccohesity, refractive index, percent transmittance, pH and osmolarity.

Keywords: Nanoemulsions; nanoemulsion characterization; entrapment efficiency; droplet size; polydispersity

Introduction:

Nanoemulsions, also known as submicron emulsions, ultrafine emulsions and miniemulsions, are submicron sized colloidal particulate systems considered as thermodynamically and kinetically stable isotropic dispersions, which consist of two immiscible liquids like water and oil, stabilized by an interfacial film consisting of a suitable surfactant and co-surfactant to form a single phase. A number of surfactants with diverse characteristics (ionic or non-ionic) had been used with such nanoemulsions. Most widely used among them were nonionic surfactants (sorbitan esters, polysorbates), anionic surfactants (potassium laurate, sodium lauryl sulphate), cationic surfactants (quaternary ammonium halide) and zwitterions surfactants (quaternary ammonium halide). Early nanoemulsions were oil-in-water (O/W) type emulsions with average droplet diameter ranging from 50 to 1000 nm. Nanoemulsions more recently are classified into three categories such as O/W type (oil is dispersed in aqueous phase), water-in-oil (W/O) type (water is dispersed in oil phase), and bi-continuous (microdomains of water and oil are interdispersed within the system). Transformation among these three types can be attained by altering the components of the emulsions. Multiple emulsions are also a type of nanoemulsions, where both O/W and W/O emulsions present simultaneously in one system. For stabilizing these two emulsions, both hydrophilic and lipophilic surfactants are used simultaneously. Nanoemulsions offer various advantages over other dosage forms and these advantages are, (1) increased rate of absorption, (2) reduced variability in absorption, (3) protection from oxidation and hydrolysis in O/W nanoemulsions, (4) delivery of lipophilic drugs after solubilisation, (5) aqueous dosage form for water insoluble drugs, (6) enhanced bioavailability for many drugs, (7) ability to incorporate both lipophilic and hydrophilic drugs, (8) delivery systems to enhance efficacy while reduce total dose and side effects, (9) as non-toxic and nonirritant vehicles for skin and mucous membrane delivery and (10) release control by permeation of drug through liquid film, whose hydrophilicity or lipophilicity as well as thickness can be precisely controlled.

Formulation of Microemulsions

A number of techniques had been adopted for formulation of nanoemulsions such as high pressure homogenization, microfludization, phase inversion, spontaneous emulsification, solvent evaporation and hydrogel formation [1-4]. Multiple emulsions are usually prepared using the double emulsion-solvent evaporation technique. A variety of techniques had been utilized for characterization of such nanoemulsions used as drug delivery systems. Nanoemulsions are formulated mainly using two primary methods, (a) the persuasion method and (b) the Brute force method.

Persuasion method/phase inversion technique

Nanoemulsion preparation by persuasion method doesn't require any external force, but instead it involves formation of fine dispersions when phase transitions occur by changing either the temperature or composition while keeping the alternate parameter constant. Persuasion method can be broadly categorised as, (i) phase transition from near-optimum state via change in single variable, which includes altering one variable of formulation such as temperature or salinity close to optimal value. Hydrophilic-lipophilic deviation (HLD) for optimal value is close to centre level for a system, for example, employing higher temperature to microemulsion. (ii) Phase transition from nearoptimal state via change in multiple variables, meaning altering more than one variable of formulation. For example, employing higher temperature and including an additional salt in a microemulsion. (iii) Catastrophic inversion, an inversion of low internal phase emulsion so that the internal phase converts to external phase. (iv) Phase transition stabilized by liquid crystal formation, which includes nanodroplets stabilization from a state close to HLD-0 by liquid crystal formation.

Brute force method

This method includes utilization of brute forces for breaking the oil droplets into the nano range. Instruments that have been utilized for formulation of nanomeulsions include high pressure homogenizer, high speed mixer, small pore membrane and high frequency ultrasonic device. Nanoemulsion properties like its small size, optical transparency and high kinetic stability is not only dependent upon the composition of variables but also on the processing variables like emulsification time, degree of mixing, energy input and emulsifying path. High-pressure homogenization and microfluidization methods are employed at both industrial and laboratory scale for attaining very small size of nanoemulsion by utilizing high pressure equipment. Various other methods are also being employed for preparation of nanoemulsion such as ultrasonication and *in situ* emulsification. Various techniques employed for preparation of nanoemulsion are shown in Table - 1

Technique	**Formulation**	**Conclusions**	**References**
High pressure homogenization	Oral lipid nanoemulsion (primaquine)	Enhanced oral bioavailability, 10-200 nm particle size	[5]
Pseudoternary phase diagram+spontaneous emulsification method	Ramipril nanoemulsion	Increased bioavailability, droplet size 80.9 nm	[6]
High pressure homogenization	O/W nanoemulsions	Improved skin hydration and elasticity	[7]
Spontaneous emulsification	O/W nanoemulsion (aceclofenac)	Nanoemulsion with potential for transdermal delivery of aceclofenac	[8]
Spontaneous emulsification	Celecoxib nanoemulsion	Enhanced physical and chemical stability of celecoxib in nanoemulsion	[9]
High pressure homogenization	Lecithin-based nanoemulsions (progesterone)	Improved permeation rates of progesterone with long-term stability	[10]
High pressure homogenization	Prednicarbate nanoemulsion	Increased chemical stability of the drug in formulation	[11]
Phase inversion temperature method	Acyclovir-loaded multiple W/O/W nanoemulsions	Excellent physicochemical stability for 6 mo at RT, mean droplet size of 100 nm	[12]
Spontaneous nanoemulsification method	Clotrimazole nanoemulsion	Improved solubility of clotrimazole, mean globule size <25 nm	[13]
Ultrasonic emulsification method	Basil oil nanoemulsion	Nanoemulsions with droplet size of 29.6 nm, for food preservation	[14]
Phase inversion composition method	Efavirenz nanoemulsion	Enhanced bioavailability, globule size <30 nm	[34]
High-pressure homogenizer	Dimethyl silicone dry nanoemulsion inhalation	Effective in acute lung injury, particle size of 19.8 nm	[15]
High-pressure homogenizer	Parenteral lecithin-based nanoemulsions (risperidone)	Enhanced brain availability of risperidone with a mean particle size of 160 nm	[28]
Microfluidization method	Pitavastatin-containing nanoemulsions	Enhanced permeation	[16]
High-pressure homogenization+ ultrasound	Nanoemulsion	Reduced energy demand for emulsification, low particle	[17]

Technique	Formulation	Conclusions	References
		dimensions and higher stability	
Sonication method	Saponin-stabilized quercetin-loaded o/w nanoemulsion	Stable for 45 d at RT, mean particle size of 52±10 nm	[18]
High-pressure homogenization	Paclitaxel-baicalein nanoemulsion	Strategy to overcome multidrug resistance	[19]
Nanoemulsion templating	PLGA nanoparticles	Imaging agents for biomedical purposes	[20]
Spontaneous emulsification method	Chitosan films with cinnamaldehyde nanoemulsions	Good UV barrier properties	[21]

Table 1: Techniques Employed for Preparation of Nanoemulsions

High pressure homogenization

Nanoemulsion preparation required high shear force, therefore in this strategy high-pressure homogenizer or piston homogenizer is utilized for production of nanoemulsions with very small particle size (up to 1 nm). In this technique, a mixture is forced to pass through an orifice at a very high pressure ranging from 500 to 5000 psi. The resultant product is further subjected to intense turbulence and hydraulic shear resulting into emulsion with extremely fine particles. This has been proved to be the most efficient method for nanoemulsion preparation but the only drawback associated with this technique is high energy consumption and rise in temperature of emulsion during processing. For obtaining smaller particle size, it also requires larger runs of homogenization cycles. Yilmaz *et al.* formulated phytosphingosine O/W nanoemulsions by employing high pressure homogenization method and found out that droplet size was decreased after 8 homogenisation cycles and such nanoemulsion was stable for over 6 mo [22].

Microfluidization

This method employed a device known as microfluidizer that utilizes high pressure positive displacement pump (500-20 000 psi) that pushes the product out through the interaction chamber consisting of stainless steel microchannels on the impingement area resulting into formation of very small particles of sub-micron range. The mixture is repeatedly circulated through the microfluidizer until the required particle size is achieved. Resultant product is also passed through the filter to separate smaller droplets from larger ones and to obtain a uniform nanoemulsion. Uluata *et al.* fabricated octadecane O/W nanoemulsions using a microfluidizer and observed that on increasing the number of passes and homogenization pressure, the droplet size decreased [23]. Goh *et al.* prepared tocotrienol-rich fraction nanoemulsions by two step homogenization where a primary coarse emulsion was prepared by using a stirrer, which was further processed using a microfluidizer. They reported that the droplet size reduced from 120 to 65.1 nm after passing through 10 homogenization cycles at an increased pressure [24].

Ultrasonication

In this technique premixed emulsion is exposed to agitation at ultrasonic frequency of 20 kHz reducing the droplets to nanodroplets size. The resultant emulsion is then passed through high shear region to form droplets with uniform size distribution. Water jacket is employed in this technique to regulate the temperature. Sonotrodes also known as sonicator probe consisted of piezoelectric quartz crystals as the energy providers during ultrasonic emulsification. On application of alternating electric voltage, these sonotrodes contract and expand. Mechanical vibrations are produced when the sonicator tip contacted the liquid resulting in cavitation, which leads to collapse of vapour cavities formed within the liquid. This technique is mainly adopted when droplet size less than 0.2 μ is required. Shi *et al.* formulated emodin-loaded nanoemulsion by using ultrasonic emulsification method at a frequency of 25 kHz and achieved mean diameter of emodinloaded nanoemulsion was found to be in the range of 10-30 nm [25].

Spontaneous emulsification

This technique involved preparation of nanoemulsion in 3 stages. The first stage included formation of an organic solution, comprising of oil and lipophilic surfactant in water miscible solvent and hydrophilic surfactant and then the O/W emulsion is formed by injecting this organic phase into the aqueous phase under magnetic stirring. The organic solvent was then removed in the third stage by evaporation. Sugumar *et al.* formulated stable eucalyptus oil nanoemulsion by adopting spontaneous emulsification and the mean droplet size was found to be in the range of 50-100 nm [26].

Solvent evaporation technique/hydrogel method

In this technique, drug solution is prepared and emulsified into another liquid (non-solvent for drug) and then solvent is evaporated, which led to drug precipitation. High speed stirrer can be employed for regulating the crystal growth and particle aggregation. Hydrogel method is very similar the solvent evaporation method. The only difference from the solvent evaporation method is that the drug solution in this case is miscible with the drug antisolvent.

Characterization of Nanoemulsions

Determination of encapsulation efficiency

For determining the amount of drug entrapped in the formulation, weighed amount of formulation is dispersed in organic solvent by ultrasonication and the drug is extracted into suitable buffer. Drug content is estimated by analysing the extract spectrophotometrically at λ_{max} of drug after making suitable dilutions against suitable blank. The entrapment efficiency (EE) and loading efficiency (LE) of the drug can be calculated by using the following Eqns. [27], drug EE = drug content in the product obtained (mg)/total amount of drug added (mg)×100 and drug LE = drug content in the product obtained (mg)/total product weight (mg)×100. Drug content could also be determined using reverse phase high-performance liquid chromatography (HPLC) techniques. Singh et al. employed this technique for finding primaquine concentration and reported 95 % encapsulation efficiency of formulated nanoemulsion [5].

Determination of particle size and polydispersity index (PDI)

The particle size and PDI of nanoemulsions are analysed employing photon correlation spectroscopy (PCS) using Malvern Zetasizer, which monitors the variation in light scattering because of Brownian motion of particles as function of time. PCS is based on the principle that the particles with small size travels with higher velocity as compared to particles with large size. The laser beam gets diffracted by sub-micron particles present in solution. Due to diffusion of particles, rapid fluctuations in laser scattering intensity occur around a mean value at a fixed angle and this is dependent upon particle size. The calculated photoelectron timecorrelation function generates a histogram of the line width distribution that can be related to the size of particle. For measuring particle size, weighed amount of formulation is dispersed in double-distilled water for obtaining homogenous dispersion and that has to be used instantly for measuring the particle size and PDI. The PDI can range from 0 to 1, where 0 (zero) stands for monodisperse system and 1 for a polydisperse particle dispersion [28]. Đorđević *et al.* evaluated the particle size and PDI of risperidone nanoemulsion by using this method and reported mean particle size around 160 nm with mean size distribution less than 0.15 [29]. Singh *et al.* has also adopted the same technique and reported particle size of primaquine nanoemulsion in the range of 20-200 nm [5].

Determination of zeta potential

The zeta potential is a method for measuring surface charge of particles when it is placed in liquid. Zeta potential is used for predicting dispersion stability and its value depends on physicochemical property of drug, polymer, vehicle, presence of electrolytes and their adsorption. It is measured by Malvern Zetasizer instrument. For measuring zeta potential, nanoemulsion is diluted and its value is estimated from the electrophoretic mobility of oil droplets. Zeta potential of ±30 mV is believed to be sufficient for ensuring physical stability of nanoemulsion. Đorđević *et al.* obtained zeta potential around –50 mV by using Malvern Zetasizer for risperidone nanoemulsion [29].

Fourier-transform infrared spectroscopy (FTIR) spectral analysis

FTIR analysis can be carried out for the assessment of drug excipient interaction, polymerization, crosslinking as well as drug loading in the formulation. It is also used for identifying the functional groups with their means of attachment and the fingerprint of the molecule. At low temperature a molecule exists in ground state and on absorbing the radiant energy, they get excited to higher energy states. IR spectroscopy is based on determining this energy difference (ΔE) between the excited and ground states of the molecule. For performing FTIR, sample can be prepared by employing suitable method such as potassium bromide pellet method, Nujol mulls and then sample is scanned in FTIR at moderate scanning speed between 4000- 400 cm^{-1}. Srilatha *et al.* conducted FTIR studies on pure drug and glipizide nanoemulsion and reported absence of drug excipient interactions (hence compatibility of drug and excipient) as all the characteristics peaks of drug appeared at same point in formulation [30].

Morphological study of nanoemulsion

The morphological study of nanoemulsion is carried by using transmission electron microscopy (TEM). In TEM, a beam of electron is incident on a thin foil specimen and passed through it. On interacting with the specimen, these incident electrons transform into unscattered electrons, elastically scattered electrons or inelastically scattered electrons. The distance among the objective lens and the specimen and among the objective lens and its image plane regulates the magnification. The electromagnetic lenses concerted the unscattered or scattered electrons and cast them onto a screen that produce amplitude-contrast picture, a phase-contrast image, electron diffraction, or a phantom picture of distinct darkness, which is dependent upon the density of unscattered electrons. Bright field imaging at increasing magnification in combination with diffraction modes used for disclosing the size and form of nanoemulsion droplets. For performing TEM, few drops of nanoemulsion or a suspension of lyophilized nanoparticles is prepared in doubledistilled water and are placed onto holey film grid and immobilized. Excess solution has to be wicked off from the grid following immobilization and stained. The stained nanoparticles are then examined at particular voltage [31]. Singh *et al.* studied surface morphology characteristics of primaquine nanoemulsion by TEM analysis and reported spherical shape of primaquine nanoemulsion with smooth surface [5].

Atomic force microscope (AFM)

AFM is comparatively a new technique being used these days for exploring the surface morphology of nanoemulsion formulations. AFM is carried out by diluting nanoemulsions with water followed by drop coating of the diluted nanoemulsion on a glass slide. Further the coated drops are dried in oven and scanned at of 100 mV/s [32]. Drais *et al.* performed AFM study on carvedilol nanoemulsion and found that the size varied from 42 to 83 nm with good stability of the formulation [33].

In vitro drug release study

In vitro drug release studies help to estimate the *in vivo* performance of drug formulation. The *in vitro* release rate of a drug is usually studied on a USP dissolution apparatus. Nanoemulsion or dried nanoparticles containing drug equivalent to 10 mg were dispersed in buffer and then it is introduced into dialysis membrane pouches and placed in a flask containing buffer. This study is carried out at 37±0.5° and a stirring speed of 50 rpm. Sample are withdrawn at periodic intervals and each time replaced by the same volume of fresh dissolution medium. Samples are then diluted suitably and the absorbance of sample is measured spectrophotometrically at a particular wavelength. Absorbance of the collected sample is used for calculating % drug release at different time intervals using calibration curve [31]. Kotta *et al.* studied the *in vitro* drug release profile of antiHIV drug nanoemulsion using dissolution apparatus type-II and reported 80 % drug release in 6 h [34].

In vitro skin permeation studies

Keshary Chien-diffusion cell is used for investigating *in vitro* and *ex vivo* permeation studies. For performing permeation studies, abdominal skin of adult male rats weighing 250±10 g is usually employed. The rat skin is positioned between the donor and the receiver chambers of diffusion cells. Temperature of receiver chambers containing fresh water with 20 % ethanol is fixed at 37° and the contents of the chamber are continuously stirred at 300 rpm. The formulations are kept in the donor chamber. At specific time intervals such as 2, 4, 6, 8 h, a certain amount (0.5 ml) of the solution from the receiver chamber was removed for performing gas chromatographic analysis and each time replaced with an equivalent volume of fresh solution immediately. Each sample is performed three times. Cumulative corrections are done for obtaining total amount of drug permeated through rat skins at each time interval and are plotted against function of time. Slope of plot is used for calculating the permeation rates of drug at a steady-state [35]. Harwansh *et al.* used Franz diffusion cell for assessing transdermal permeability of glycyrrhizin through human cadaver skin and reported increased permeability with nanoemulsion formulation as compared to conventional gel [36].

Stability studies

Stability studies are performed for assessing stability of the drug substance under the influence of a various environmental factors like temperature, humidity and light. The stability studies of nanoemulsion are carried out after storing the formulation for 24 mo in dispersed and freeze-dried state as per International Conference on Harmonisation guidelines. The storage conditions followed are ambient (25±2°/60±5 % RH), refrigeration (5±3°) and freeze (–20±5°). The requisite volume of nanoemulsion is stored in glass bottles and is tightly sealed. Samples are withdrawn at predefined time interval and analysed for the characteristics such as particle size, loading and EE and *in vitro* drug release profile [26]. Singh *et al.* performed stability studies on nanoemulsion and observed that no change in viscosity, drug content and particle size when the formulation was stored for 3 mo at 25°/60 % RH and 30°/65 % RH [5].

Shelf life determination

For determining shelf life of a nanoemulsion, accelerated stability studies are performed. The formulations are stored at three distinct temperatures and ambient humidity conditions (30°, 40° and 50±0.5°) for almost 3 mo. After a particular time interval (0, 30, 60 and 90 d) samples are withdrawn and analysed using HPLC at λ_{max} for estimating the remaining drug content. Samples withdrawn at zero time are used as controls. The order of the reaction is determined by this and after that the reaction rate constant (K) for the degradation is calculated from the slope of the lines by using following equation at each elevated temperature: slope = $-K/2.303$, the logarithm values of K are plotted at different elevated temperatures against the reciprocal of absolute temperature (Arrhenius plot). From this plot value of K at 25° is determined and it is further used for calculating shelf life by putting the value in following Eqn.: $t_{0.9}=0.1052/K_{25}$. Where $t_{0.9}$ stands for time required for 10 % degradation of the drug and it is termed as shelf life [31]. Ali *et al.* determined the shelf life of clobetasol propionate-loaded nanoemulsion around 2.18 y at room temperature (25°) and concluded that the stability of clobetasol propionate can be augmented by incorporating in a nanoemulsion [37]. Parveen *et al.* reported that the shelf life of a silymarin nanoemulsion to be around 3.8 y when stored in a refrigerator [38].

Thermodynamic stability studies

Thermodynamic stability studies are usually carried out in three steps. Firstly heating-cooling cycle, which is performed for observing any effect on the stability of nanoemulsion by varying temperature conditions. Nanoemulsion is exposed to six cycles between 4° (refrigeration temperature) and 40° by storing the formulation at each temperature for not less than 48 h. The formulations which are stable at these temperatures are further chosen for centrifugation studies. Secondly, centrifugation study in which the formulated nanoemulsions are centrifuged at 5000 rpm for 30 min and observed for phase separation or creaming or cracking. Those which did not show any sign of instability are subjected to freeze thaw cycle. Thirdly, the freeze-thaw cycle, in which nanoemulsion formulations are exposed to three freeze-thaw cycles with temperature varying between –21° and +25°. Formulations that show no signs of instability pass this test and deemed to have good stability [6]. These formulations are then subjected to dispersibility studies for evaluating the efficiency of self-emulsification. Srilatha *et al.* performed thermodynamic studies on glipizide nanoemulsion by subjecting it to three cycles of stability and reported good physical stability of nanoemulsion with no appearance of phase separation, creaming or cracking [30].

Dispersibility studies

Dispersibility studies for evaluating the efficiency of self-emulsification of nanoemulsion are carried out by using a standard USP XXII dissolution apparatus 2.1 ml of each formulation is incorporated into 500 ml of distilled water maintained at 37±0.5°. A standard stainless steel dissolution paddle rotates at 50 rpm for providing gentle agitation. In vitro performance of the nanoemulsion formulations is evaluated visually by using a grading system described below[6]. Grade A nanoemulsions form rapidly within 1 min and appear to be clear or bluish. Grade B nanoemulsions form rapidly but are slightly less clear emulsions appear to be bluishwhite. Grade C nanoemulsions are fine milky emulsion that form within 2 min. Grade D are those dull, greyishwhite emulsions that has a little oily appearance and are slower to form (>2 min). Grade E nanoemulsions display either poor or negligible emulsification with large oil globules present on the surface.

Determination of viscosity

Viscosity assessment is an important parameter for physicochemical characterization of nanoemulsion. Various instruments are employed for measuring viscosity such as Ostwald viscometer, Hoeppler falling ball viscometer, Stormer viscometer, Brookfield viscometer and Ferranti-Shirley viscometer. Among all these viscometer, Brookfield is the preferred one for measuring the viscosity of nanoemulsion. Determination of viscosities affirms whether the system is O/W or W/O emulsion. Low viscosity of systems shows that it is O/W type and high viscosity shows that it is water in oil type system [28]. However, currently survismeter has been the most widely employed equipment as it measures surface tension, viscosity, interfacial tension, contact angle, dipole moment and particle size and hydrodynamic volumes of the nanoemulsions [39]. Shafiq *et al.* has determined viscosity of ramipril nanoemulsion formulations by using Brookfield cone and plate rheometer and reported the viscosity of formulations as less than 21 cP with the minimum viscosity of 10.68 cP [6].

Refractive index

Refractive index tells how light propagates through the medium and transparency of nanoemulsion. Refractive index (n) of medium can be defined as ratio of speed of wave (c) in reference medium to the phase speed of wave (vp) in medium: n=c/vp. Refractive index of the nanoemulsion can be determined by Abbes type refractometer at 25±0.5° by placing a drop of nanoemulsion on slide and comparing it with refractive index of water (1.333). If refractive index of nanoemulsion has equal refractive index as that of water, then the nanoemulsion is considered to have transparent nature [2,28]. Harika *et al.* measured the refractive index of amphotericin B nanoemulsion by Abbe refractometer and the value of refractive index of the formulation was found to be similar to that of the water [40].

Percent transmittance

Percent transmittance of a formulated nanoemulsion is estimated using UV spectrophotometer at a particular wavelength with distilled water as a blank. If percent transmittance of a nanoemulsion is found to be greater than 99 %, then it is considered as transparent in nature [31]. Harika *et al.* reported percent transmittance of >97 % for a amphotericin B nanoemulsion formulated [40].

pH and osmolarity measurements

The pH meter is used for measuring the pH of a nanoemulsion and microosmometer is used for determining the osmolarity of emulsion, which is based upon freezing point method. For performing this, 100 µl of nanoemulsion is transferred in microtube and measurements are taken [41]. Morsi *et al.* measured the pH of the acetazolamide nanoemulsion by pH meter and found pH in the range of 4.9 to 5.5 thus claiming it to be adequate and non-irritant for application to the eye [42].

Dye solubilisation

A water soluble dye is dispersible in an O/W globule whereas it is soluble in the aqueous phase of the W/O globule. Similarly an oil soluble dye is dispersible in the W/O globule but soluble in the oily phase of the O/W globule [3]. On adding water soluble dye to O/W nanoemulsion, it will evenly takes up the colour whereas if it is a W/O emulsion, dye will remain in dispersed phase only and the colour will not spread evenly. This can be seen with microscopic examination of emulsion [4]. Laxmi *et al.* carried out this test on artemether nanoemulsion by adding eosin yellow, a water soluble dye to the formulation and examined it under a microscope. They discovered that the aqueous continuous phase was labelled with dye while the oily dispersed phase remained unlabelled therefore confirming the formed nanoemulsion as O/W type [43].

Dilutability test

The rationale of dilution test is that continuous phase can be added in larger proportion into a nanoemulsion without causing any problem in its stability. Thus O/W nanoemulsions are dilutable with water but W/O nanoemulsions are not and go through a phase inversion into O/W nanoemulsion. The W/O nanoemulsion can be diluted with oil only [3,4].

Laxmi *et al.* performed dilutability test on nanoemulsion by diluting it with water and observed no sign of phase inversion and precipitation thus claiming their nanoemulsion formulation to be stable [43].

Conductance measurement

The O/W nanoemulsions are highly conducting because they have water in external phase whereas W/O nanoemulsions are not conducting as they have water in internal or dispersal phase. Electrical conductivity measurements are very much beneficial for determining the nature of the continuous phase and for detecting phase inversion phenomena. At low volume fractions, increase in conductivity of certain W/O nanoemulsion systems was observed and such kind of behaviour is deduced as an indicator of a percolative behaviour or ions exchange among droplets prior to the development of bicontinuous structures. Dielectric measurements are a great means of exploring the structural and dynamic features of nanoemulsion systems [3]. Conductometer is employed for determining the conductance of nanoemulsion. For carrying out conductance measurement, a pair of electrodes is attached to a lamp and an electric source is immersed into an emulsion. When the emulsion is O/W type then water will conduct the current and lamp will glow because of passage of current among connecting electrodes. The lamp will not glow if it is water in oil emulsion as oil in external phase does not conduct the current [4]. Harika *et al.* performed conductivity test on amphotericin B nanoemulsion using an electroconductometer. They reported conductivity of the formulations in the range of 454.2-552.3 µS/cm and concluded the system to be O/W on the basis of electroconductivity study [40].

Interfacial tension

By measuring the interfacial tension, the formation and the properties of nanoemulsion can be investigated. Ultra low values of interfacial tension corresponds to phase behaviour, mainly the coexistence of surfactant phase or middle-phase nanoemulsions with aqueous and oil phases in equilibrium. For determining ultralow interfacial tension spinning-drop apparatus is used. Interfacial tensions are obtained by measuring the shape of a drop of the low-density phase, rotating it in cylindrical capillary filled with high-density phase [3].

Fluorescence test

There are numerous oils that show fluorescence under UV light. If a W/O nanoemulsion is subjected to a fluorescence light under a microscope, the whole field will fluoresces and if it is an O/W the fluorescence will be in spots [4].

***In vivo* studies**

In vivo studies can be performed by adopting suitable animal model according to the activity chosen. Srilatha *et al.* has performed antidiabetic activity on glipizide nanoemulsion by choosing hyperglycaemia model in which they first induce diabetes in rats by intraperitoneal injection of streptozotocin solution and then the formulation was given to diabetic rats and the pharmacodynamic studies were performed on them. They reported the reduction in blood glucose levels for up to 12 h [30]. Chouksey *et al.* has evaluated *in vivo* performance of atorvastatin nanoemulsion by performing pharmacokinetic studies on nanoemulsion and they reported better bioavailability of nanoemulsion formulation as compared to pure drug [44].

Nanoemulsions hold great potential as an efficient drug delivery tool that could be effectively harnessed to realise the complete potential. Quality assurance and quality control shall be of paramount importance with such a precise delivery system and hence the evaluation tests are to be performed rigorously.

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