ENHANCEMENT OF DISSOLUTION CHARACTERISTICS OF CLOPIDOGREL BISULPHATE BY PRONIOSOMES

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ABSTRACT

Objective: The present investigation aims to formulate and evaluate proniosomes of clopidogrel bisulphate for improving its dissolution characteristics.

Methods: The slurry method was used for the preparation of proniosomes of clopidogrel using cholesterol, sorbitan monostearate (Span 60) and maltodextrin as a carrier. Clopidogrel proniosomes were evaluated for their entrapment efficiency and in vitro drug release. The best formula (F1) that achieved maximum drug release was further evaluated by measurement of the angle of repose, morphological examination, determination of vesicle size, determination of zeta potential, Fourier transform infrared spectroscopy and differential thermal analysis. The in vivo behavior of the selected proniosomal formula (F1) was studied by measuring the antiplatelet activity in adult male mice.

Results: The entrapment efficiency of clopidogrel proniosomes was in the range of 83.04±1.99 to 90.14±0.30 % drug released from proniosomal formulations was in the range of 79.73±0.35 to 97.70±1.0 % within 4 h. Clopidogrel proniosomes significantly enhanced the in vitro release of clopidogrel compared with the plain drug that achieved 61.77±2.22 % drug release. F1 significantly (p ≤ 0.001) increased the bleeding time and prolonged prothrombin time and decreased prothrombin activity and increased the international normalized ratio (INR) compared to plain clopidogrel.

Conclusion: The present investigation introduced proniosomes as a promising carrier for clopidogrel that could enhance its dissolution and pharmacological effect.

Keywords: Clopidogrel, Maltodextrin, Proniosomes, Pharmacological effect

INTRODUCTION

Clopidogrel bisulphate is a weak base that is known chemically as Methyl (S)-α-(2-chlorophenyl)-6,7-di-hydrothieno[3,2-c]pyridine-5(4H)-acetate sulfate. Clopidogrel is a potent antiplatelet agent that is widely used for the prevention of coronary artery diseases and treatment of vascular thrombosis. However, it is of low aqueous solubility and therefore, it has low oral bioavailability (less than 50%). Enhancement of solubility is of critical concern in improving dissolution rate and bioavailability. Recently, different nanotechnology techniques have been used to increase the dissolution rate and bioavailability of many drugs [1, 2].

Liposomes are phospholipid nano-vesicles that are used as drug carriers, but they suffer from several disadvantages like physical and chemical instability [3, 4]. Therefore, special storage conditions and handling are required [5]. Niosomes are non-ionic surfactant vesicles that protect the drug against enzymatic degradation and improve drug permeability by their surface-active properties [6]. Niosomes are more stable chemically than liposomes, but they are also physically unstable due to the possibility of aggregation, fusion, and leakage of vesicles. Proniosomes are free-flowing powder form of niosomes [7]. Proniosomes could overcome physical instability problems of niosomes [8] and offer better convenience of storage and transportation. Following hydration, proniosomes are converted to niosomes [9, 10].

Though the oral drug delivery is the most common and preferred route of drug administration, failure of the conventional delivery of many drugs may happen due to poor drug absorption and highly fluctuated drug plasma levels. Lipid nano-vesicles like proniosomes have various advantages over conventional drug delivery systems because they act as drug reservoirs that enhance both drug solubilization and permeation. Moreover, proniosomes provide controlled drug release to deliver the effective dose of the drug to its target site [8, 9].

The aim of this study is to formulate proniosomes of clopidogrel by the slurry method for enhancement of dissolution and pharmacological effect of clopidogrel.

MATERIALS AND METHODS

Materials

Clopidogrel was a gift sample from the Egyptian International Pharmaceutical Industries Co., EPICO, Egypt. Cholesterol and maltodextrin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium dihydrogen phosphate and sodium hydroxide were purchased from Merck KGaA (Darmstadt, Germany). Sodium chloride was purchased from Carlo Erba (Milano, Italy). Sorbitan monostearate (Span 60) was purchased from Acros Organics (Geel, Belgium). Clopidogrel bisulfate USP and clopidogrel hydrobromide USP were purchased from Sigma Aldrich (St. Louis, MO, USA). The commercial formulation bisulfate clopidogrel tablets (Plavix, Sanofi-Aventis, USA) was used as a reference sample. All other solvents and reagents were of analytical grade.

Methods

Preparation of clopidogrel proniosomes

Clopidogrel proniosomes were formulated by the slurry method using maltodextrin as a carrier, table 1. Accurately weighed amounts of clopidogrel, Span 60 and cholesterol were dissolved in chloroform/ethanol mixture (2:1 v/v). The resultant solution is added to a round-bottom flask containing maltodextrin carrier to form the slurry. The organic solvents were removed using a rotary evaporator (Buchi rotavapor R-3000, Switzerland) that was adjusted to 70 rpm, at 45±2 °C under reduced pressure until the mass in the round-bottom flask become dry free-flowing product [13]. The formulated proniosomes were stored in well-closed containers to be used for further studies.
efficiency was calculated as follows:

$$EE(\%) = \left( \frac{A1 - A2}{A1} \right) \times 100/A1$$

Where $A$ is the Initial amount of drug, $A2$ Amount of drug in the supernatant

\[\text{In vitro release of clopidogrel proniosomes}\]

In vitro release of clopidogrel proniosomes and aqueous drug dispersion was performed using modified Franz diffusion cells. Cellulose membrane was mounted between donor and receptor compartments. The receptor medium was phosphate buffer solution (PBS) pH 6.8 (containing 1% sodium lauryl sulphate) that maintained at 37 °C±0.5 °C and stirred at 50 rpm. The proniosomal formulations and aqueous drug dispersion were loaded on the donor compartment. Samples were withdrawn at different time intervals and replaced by the same amount of fresh buffer. The drug concentrations were determined using UV spectrophotometer. Triplicate experiments were performed. The results were expressed as mean values ± SD [2, 16].

The similarity factor test was used to compare the overall dissolution profiles and to determine the similarity in the pattern of drug release of plain drug and different proniosomal formulations [17]. The two preparations are said to be similar if the value of $f_2$ lies between 50 and 100. $f_2$ is calculated by the formula given below:

$$f_2 = 50.0 \times \log \left[ 1 + \frac{1}{n} \sum_{i=1}^{n} \left( \frac{R_i - T_i}{R_i} \right)^2 \right]^{0.5}$$

Where, $R_i$ and $T_i$ are percentages of drug dissolved at a specified time from the standard and test formulations respectively, $n$ no of sampling points.

\[\text{Kinetic treatment}\]

The release kinetics and mechanism of the in vitro release of clopidogrel proniosomes were studied by treating the data of drug release using different mathematical models as zero-order, first-order kinetics and Higuchi model [18].

Zero-order model: $A = K_o t$

First-order model: $\ln (1 - A) = - K_t t$

Higuchi model: $A = KH^{1/2}$

Where: $A$ is the fraction of drug released in time $t$, $K_r$ is the rate constant of zero-order, $K$ is the rate constant of first-order and $K_h$ is the rate constant of Higuchi diffusion model.

\[\text{Evaluation of the selected formula of clopidogrel proniosomes}\]

Clopidogrel proniosomal formulation that achieved the best in vitro release was evaluated by measurement of the angle of repose, Fourier-transform infrared spectroscopy (FTIR), differential thermal analysis (DTA), morphological examination and determination of vesicle size and zeta potential.

\[\text{Micromeric properties}\]

The flow properties of the chosen proniosomal powder were determined by measurement of the angle of repose. The angle of repose of both maltodextrin and the chosen proniosomal powder was determined by the funnel method. A funnel was fixed at a certain height so that the outlet orifice of the funnel is 5 cm above a graph paper placed on a horizontal surface. Maltodextrin or proniosome powder was flowed down through the funnel until the apex of the powder pile just touched the funnel tip [19]. The angle of repose was estimated as follows:

$$\tan \theta = h/r$$

($\theta$ is the angle of repose, $h$ is the height of the powder pile and $r$ is the radius of powder pile).

\[\text{Photomicroscopic examination}\]

The formation of the niosomal vesicles upon hydration of the proniosomal powder was investigated by photomicroscopic examination. The selected proniosomal powder was spread on a glass slide and few microliters of distilled water (80 °C±2 °C) were added dropwise. The morphology of the selected proniosomal powder (F1) and formation of the niosomal vesicles were observed under a light microscope (Coslabs micro, India) [8, 17].

\[\text{Scanning electron microscopy (SEM)}\]

The morphology of the chosen proniosomal formula derived niosomes was examined by the scanning electron microscope (SEM) (Jeol, JSM-6360, Japan). The optimized dispersion was firstly diluted with distilled water and dropped on the copper mesh. Few minutes after drying of excessive circumjacent liquid, 1% phosphotungstic acid was added for a few minutes as a negative stain. The over flowed solution was sipped up and dried in the air [18, 19].

\[\text{Vesicle size and zeta potential determination}\]

Determination of vesicle size and zeta potential was performed for investigating the colloidal characteristics of the chosen proniosomal formula (F1). The selected proniosomal formula was hydrated with distilled water (80 °C±2 °C) for the formation of the niosomal vesicles. The niosomal dispersion was suitably diluted by distilled water. Then, laser scattering particle size analyzer (MALVERN ZETASIZER, MAL500099) was used for estimation of vesicle size and zeta potential [23].

\[\text{Fourier transform infrared spectroscopy (FT-IR)}\]

Fourier transform infrared spectroscopy was performed using FTIR spectrometer (FT-IR Shimadzu 6300 Japan). Samples of clopidogrel, Span 60, cholesterol, maltodextrin, plain proniosomes and the

\[
\begin{array}{|c|c|c|}
\hline
\text{Formulation Code} & \text{Ratio(umol)} \text{(Cholesterol: span60)} & \text{Maltodextrin (mg)} \\
\hline
F1 & 40: 210 & 210 \\
F2 & 60: 190 & 190 \\
F3 & 80: 170 & 170 \\
F4 & 100: 150 & 150 \\
F5 & 120: 130 & 130 \\
F6 & 140: 110 & 110 \\
F7 & 160: 90 & 90 \\
\hline
\end{array}
\]

\*Each formulation containing clopidogrel bisulphate 98 mg (which is equivalent to 75 mg of clopidogrel base), 1 g of Carrier (maltodextrin) per 1 mmol of Span 60
chosen clopidogrel proniosomal formula were used. All these samples were grounded and mixed with potassium bromide to form KBr pellets in a hydraulic press (Kimaya Engineers, Maharashtra, India). The scanning range was 4000–400 cm⁻¹ using PerkinElmer instrument (Spectrum RXI-5.31, UK) [1, 21].

**Differential thermal analysis (DTA) study**

DTA was used to investigate the change in the crystalline state of clopidogrel in the chosen proniosomal formula using differential thermal analyzer (Shimadzu DTA–50 module, Kyoto, Japan). The rate of heating was 10 °C/min. The samples of clopidogrel, Span 60, cholesterol, maltodextrin, plain pronosomes, and the chosen clopidogrel proniosomal formula were heated in the range of 30–400 °C under dry nitrogen flow rate of 15 ml/min [22, 23].

**In vivo study**

**Animals**

White male albino mice weighing 20–30 gm were used in this experiment. Mice were purchased from the National Research Center (Dokki, Giza, Egypt) and housed in a pathogen-free facility in sawdust bedded cages. The animal rooms were maintained at [25±2] °C with 50% relative humidity and a 12 h light: dark cycle. All the tested mice were acclimatized under standard conditions in the animal house for at least two weeks prior to the experiments. The study protocol and animal use were approved by The Ethical Committee of Faculty of Pharmacy, Kafershelkheit University. All the experimental procedures were performed in accordance with the recommendations of the ARRIVE guidelines [27] and comply with European Union Directive as 2010/63/EU [28] and the U. K. Animals (Scientific Procedures) Act, 1986 (ASPA) [29].

**Experimental protocol**

Mice were divided into 3 groups (10 mice each) the first group was treated with an aqueous dispersion of clopidogrel bisulfate (20 mg/kg) as a single oral dose (Standard group). The second group was gavaged with a single dose of clopidogrel proniosomal formula (F1) equivalent to 20 mg/kg orally (Test group). The third group was served as the control group. Mice in this group received only distilled water. Five mice of each group were used to evaluate platelet aggregation through measuring bleeding time and bleeding volume. The other five mice were integrated into the evaluation of coagulation activity markers [30].

**Platelet aggregation inhibition assay**

This study was done for studying the platelet aggregation inhibition effect of clopidogrel bisulfate and the selected clopidogrel proniosomal formula (F1). Five mice of each group were anesthetized using diethyl ether after 1h of treatment. The tails of the anesthetized mice were cut (about 0.5 cm from the distal end of the tail) then embedded in tubes containing 2 ml of phosphate saline buffer (PH 7.4 at 37 °C). The time until the bleeding stopped was detected using a stopwatch. The collected blood volume was then measured. The bleeding time and the bleeding volume were recorded 1h and 3 h after oral administration of the plain drug and the selected clopidogrel proniosomal formula (F1) [31].

**Detection of coagulation profile**

3 h after oral administration of clopidogrel and the selected clopidogrel proniosomal formula (F1), all mice were anesthetized with diethyl ether. Blood samples were collected via cardiac puncture and collected into tubes containing sodium citrate. The citrated plasma was used to evaluate the prothrombin time and activity. Assay of prothrombin time and activity were done according to kits’ instructions (EGY-CHEM for lab technology, Badr City, Egypt). Briefly, normal citrated plasma was added to tissue thromboplastin. The extrinsic clotting mechanism is then initiated forming a solid gel clot within a specified period of time. The test was made in triplicate and the results are the mean of three measurements. The international normalized ratio (INR) was calculated as the ratio between the mean of the prothrombin time of mouse plasma in seconds and mean of normal prothrombin time (MNPT) [29, 30].

**Statistical analysis**

Statistical analysis was performed using one-way analysis of variance (ANOVA) using Graph Pad Prism version 7 software. The differences were considered to be significant if P<0.05.

**RESULTS AND DISCUSSION**

**Preparation of clopidogrel proniosomes**

Clopidogrel Proniosomes were prepared by the slurry method. Span 60 was chosen as the non-ionic surfactant that facilitates the formation of stable vesicles as it has a high phase transition temperature. Moreover, Span 60 could enhance the oral delivery of clopidogrel from the proniosomal vesicles [34]. Cholesterol was used to improve the encapsulation efficiency and stability of the prepared vesicles. Maltodextrin was chosen as the coating carrier [15]. Solvents used were chloroform and ethanol (2:1) because clopidogrel and lipids are soluble in this mixture and also ethanol is less toxic than methanol [11]. The ratio of cholesterol to Span 60 was changed from 40: 210 to 160: 90 while keeping the total lipid constant at 250 µM [13].

**Preparation of niosomes from clopidogrel proniosomes**

Clopidogrel niosomes were easily and directly formed after hydration of proniosome powder with distilled water at 80±2 °C. Vesicular structures were formed over the surface of maltodextrin that may be attributed to swelling of the lipid bilayer that transformed into multilamellar vesicular structures with gentle agitation [15]. The multilamellar vesicles are further converted to unilamellar niosomes by sonication.

**Determination of entrapment efficiency of clopidogrel proniosomes**

The entrapment efficiency of clopidogrel proniosomes was in the range of 83.04±1.99 to 90.14±0.30, table 2. High values of encapsulation efficiency may be attributed to the poor water solubility of clopidogrel and its dissolving in the lipid bilayers of niosomal vesicles [32, 33].

F4 showed the highest encapsulation efficiency of 90.14±0.30%. As the ratio of cholesterol to Span 60 increased, the entrapment efficiency increased. That may be explained by the ability of cholesterol to increase the bilayer rigidity by intercalation between the bilayers of non-ionic surfactants [37]. However, very high cholesterol concentration was found to be of a lowering effect on the entrapment efficiency of the drug. This could be due to the fact that cholesterol beyond a certain level has the ability to disrupt the regular bilayered structure leading to decreasing drug entrapment [13]. Moreover, that may be attributed to competition between clopidogrel and cholesterol for packing in the limited sites in the bilayered structure of proniosomal vesicles thus excluding clopidogrel and disrupting the well-ordered bilayered structure [35, 36].

| Formulation code | Entrapment efficiency |
|------------------|-----------------------|
| F1               | 83.04±1.99            |
| F2               | 84.07±3.02            |
| F3               | 88.45±0.20            |
| F4               | 90.14±0.30            |
| F5               | 89.72±2.00            |
| F6               | 88.23±0.23            |
| F7               | 85.70±1.10            |

*Data are expressed as mean±SD, n=3
The ANOVA analysis of entrapment efficiency of proniosomal formulations showed that there was significant difference in entrapment efficiency between different clopidogrel proniosomal formulations as the ratio of cholesterol to Span 60 changed from 40:210 to 160:90, table 3. F values were above the critical F values and that made P values <0.05 (threshold level). Thus, the null hypothesis (H0) is rejected.

| Source                          | SS  | DF | MS  | F     | P value |
|---------------------------------|-----|----|-----|-------|---------|
| Difference (between groups)     | 140.4 | 6 | 23.41 | 8.86  | 0.0004  |
| Difference (within groups)      | 36.99 | 14| 2.642 |       |         |
| Total                           | 177.4 | 20|       |       |         |

Table 3: ANOVA analysis of entrapment efficiency of clopidogrel proniosomes

**In vitro release of clopidogrel proniosomes**

From fig. 1, the in vitro release of clopidogrel proniosomal formulations was in the range of 79.73±0.35 to 97.70±1.10 within 4 h. It is observed that the results of entrapment efficiency are consistent with those of % drug released, as F1 composed of cholesterol and Span 60 (40:210) molar ratio with the lowest entrapment efficiency showed the highest % drug released. That was in good agreement with results reported by Singh et al. (2011) who concluded that niosomes that had the highest entrapment efficiency exhibited the lowest in vitro drug release due to decreasing drug efflux [36].

| Source                          | SS  | DF | MS  | F     | P value |
|---------------------------------|-----|----|-----|-------|---------|
| Difference (between groups)     | 624.5 | 6 | 104.1 | 25.36 | P<0.0001 |
| Difference (within groups)      | 57.47 | 14| 4.105 |       |         |
| Total                           | 682 | 20|       |       |         |

Table 4: ANOVA analysis of in vitro release of clopidogrel proniosomes

It is concluded that as the molar ratio of cholesterol to surfactant increased, % drug released decreased. That may be explained by a marked reduction in the efflux of the drug due to membrane stabilizing ability of cholesterol and its ability to abolish the gel-to-liquid phase transition of niosomal systems [13]. However, a further increase in the amount of cholesterol beyond certain value caused disruption of the bilayered structure of the vesicles leading to higher drug release [20]. ANOVA analysis showed that increasing the molar ratio of cholesterol to Span60 from 40:210 to 160:90 resulted in significant (p<0.001) effect on % drug released, table 4.

An aqueous dispersion of plain drug achieved 61.77±2.22 % drug release after 4 h. Higher dissolution profile obtained with the proniosomal formulations than the plain drug might be due to an enhancement of solubility of clopidogrel by the non-ionic surfactant or due to change in clopidogrel structure from crystalline to amorphous state in proniosomal vesicles [31]. Moreover, it could be attributed to adsorption of lipid coat of proniosomal vesicles on maltodextrin increasing the effective surface area [13]. ANOVA analysis showed that incorporation of clopidogrel into proniosomes resulted in a significant increase in % drug released compared to the plain drug (p<0.001), table 5.

| Source                          | SS  | DF | MS  | F     | P value |
|---------------------------------|-----|----|-----|-------|---------|
| Difference (between groups)     | 2431 | 7 | 347.3 | 82.54 | P<0.0001 |
| Difference (within groups)      | 67.32 | 16| 4.208 |       |         |
| Total                           | 2498 | 23|       |       |         |

Table 5: ANOVA analysis of in vitro release of clopidogrel proniosomes in comparison to plain drug
The dissolution profile of different proniosomal formulations was compared with plain drug using the similarity factor test, table 6. The estimated values of $f_2$ were found to be less than 50. Therefore, it is obvious that there is a significant difference in drug release between different proniosomes in comparison to clopidogrel.

Table 6: Similarity factor test of clopidogrel proniosomes in comparison to plain drug

| Formulation code | Similarity factor |
|------------------|------------------|
| F1               | 24               |
| F2               | 30               |
| F3               | 41               |
| F4               | 47               |
| F5               | 44               |
| F6               | 39               |
| F7               | 37               |

Based on the previous results, formula F1 that achieved the highest % drug release after 4h (97.70 ±1.10%) and had 83.04±1.99% encapsulation efficiency of the drug was chosen for further investigation.

Kinetic treatment

To find out the mechanism and order of drug release, data of in vitro release of clopidogrel formulations were fitted into different kinetic models, table 7. The correlation coefficient values indicated that the profile of drug release followed Higuchi diffusion model. These results indicated that the transport mechanism of the drug from clopidogrel proniosomes is by diffusion [41].

Table 7: Kinetic parameters of clopidogrel proniosomes

| Formula | Zero-order | First order | Higuchi model |
|---------|------------|-------------|---------------|
| F1      | 0.987      | 0.970       | 0.998         |
| F2      | 0.991      | 0.990       | 0.998         |
| F3      | 0.991      | 0.990       | 0.999         |
| F4      | 0.992      | 0.995       | 0.999         |
| F5      | 0.995      | 0.990       | 0.999         |
| F6      | 0.993      | 0.983       | 0.998         |
| F7      | 0.997      | 0.977       | 0.998         |

Evaluation of the selected formula of clopidogrel proniosomes

Micromeritic properties

Good flowability of powders is an important character in solid dosage forms to achieve better handling and processing. Results of micromeritic properties of maltodextrin and the selected proniosomal formulation were listed in table 8. It was observed that the angle of repose of clopidogrel proniosomal powder (31.67 °±0.49 °) was lower than that of maltodextrin (46.12 °±0.53 °). Flowability is represented by the angle of repose ($\theta$). A value of $\theta$ between 25-30 ° indicates excellent flow, a value of $\theta$ between 31–35 ° indicates good flow, a value of $\theta$ between 36–40 ° indicates fair flow, a value of $\theta$ between 41–45 ° indicates passable flow, a value of $\theta$ between 46–55 ° indicates ‘poor’ flow and a value of $\theta$ higher than 56 ° indicates ‘very poor’ flow [42]. Based on this, the flow of clopidogrel proniosomes was rated as good flow, whereas the flow of maltodextrin was rated as poor flow. These results showed that proniosomal formula is of good flowability that is higher than that of maltodextrin powder.

Table 8: Micromeritic properties of maltodextrin and selected proniosomal formula (F1)

| Formulation                  | Angle of repose |
|------------------------------|-----------------|
| Maltodextrin                 | 46.12 °±0.53 °  |
| Proniosomal formula (F1)     | 31.67 °±0.49 °  |

‘Data are expressed as mean±SD, n=3

Photomicroscopic examination

Examination of the chosen proniosomal formula under an optical microscope, fig. 2 showed that the surface of clopidogrel proniosomes was irregular due to a coating of the molecules of Span 60 and cholesterol over the surface of maltodextrin [15]. Moreover, proniosomes were progressively and rapidly converted to niosomes upon addition of the aqueous phase due to swelling of the bilayers and vesicles by an interaction of water with the hydrophilic groups of surfactant. The swollen bilayers tend to form spherical structures giving rise to multi-lamellar and multi-vesicular structures that are converted to unilamellar niosomal vesicles upon sonication.

Our results agree with those reported by Singh et al. (2012) [43].

Fig. 2: Optical photomicrograph of (a) proniosomal clopidogrel powder (10×) (b) proniosomal clopidogrel moist powder before sonication (40×), and (c) clopidogrel niosomes after sonication (40×)
Scanning electron microscopy (SEM)

The SEM micrograph of the chosen proniosomal formula is illustrated in fig. 3. It was observed that the niosomal vesicles formed by hydration of proniosomes were discrete and spherical in shape with sharp boundaries. This may be attributed to the fact that the non-ionic surfactants form a closed bilayer vesicle in water based on its amphoteric nature using some energy like physical agitation or heat [44]. Spherical shape of niosomes may be attributed to their tendency to minimize their surface free energy. That is in accordance with the results reported by Das and Palei (2011) [45].

Fig. 3: Scanning electron micrograph of hydrated proniosomes of clopidogrel (F1)

Vesicle size and zeta potential determination

The particle size distribution of the chosen proniosomal formula showed unimodal normal symmetrical frequency distribution patterns, fig. 4. The mean vesicle size of the hydrated clopidogrel proniosomes was 217.9 nm. The polydispersity index, PDI, is an important parameter that describes the vesicle size distribution and it varies from 0.0 to 1.0. As the PDI value is closer to zero, the more homogenous the vesicles size. PDI was 0.005 indicating a narrow and homogenous size distribution [46].

Fig. 4: Particle size distribution curve of clopidogrel proniosomal formula (F1)

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of clopidogrel, Span 60, cholesterol, maltodextrin, drug-free proniosomes and the chosen clopidogrel proniosomal formula (F1) were illustrated in fig. 6. The FTIR spectrum of clopidogrel showed characteristic peaks of clopidogrel which were absorption band at 2544 cm⁻¹ which is due to (N+-H), 1751 cm⁻¹ (C=O, stretching), 1190 cm⁻¹ (C-O, stretching), 1030 cm⁻¹ (C-O) [48]. Span 60 exhibited characteristic peaks at 3410 which is due to aliphatic O–H stretch, 2936 cm⁻¹ (C–H stretch) and 1745 cm⁻¹ (C =O stretch of ester). The characteristic peaks of cholesterol appeared at 3400 cm⁻¹ (OH stretching) and the observed bands between 2800–3000 cm⁻¹ are due to asymmetric and symmetric stretching vibrations of CH₂ and CH₃ groups. [49]. FTIR spectrum of maltodextrin showed an absorption band at 3399 cm⁻¹ for OH group, a strong broadband between 980 cm⁻¹ and 1200 cm⁻¹ which is the most characteristic band for polysaccharides. The peaks at 1152 cm⁻¹ and 1018 cm⁻¹ were attributed to C–O stretching [50]. IR spectrum of plain proniosomes exhibited the characteristic peaks of cholesterol, Span 60 and maltodextrin with the reduced intensity that may be due to the formation of the lipid bilayer. FTIR spectrum of the selected proniosomal formula exhibited the characteristics peaks of clopidogrel that confirmed the absence of any chemical interactions between clopidogrel and other excipients [12]. Reduced-intensity and minor shifting of the characteristic peaks of clopidogrel may be explained on the basis of formation of Van der Wall forces, hydrogen bonds or dipole-dipole interactions between clopidogrel and other proniosome contents leading to the formation of stable proniosomal vesicles with high entrapment efficiency of the drug [49].

Fig. 5: Zeta potential distribution of clopidogrel proniosomal formula (F1)

Differential thermal analysis (DTA) study

DTA could be used to detect the thermal behavior and physical state of drug. DTA thermograms of clopidogrel, Span 60, cholesterol,
maltodextrin, plain (drug-free) proniosomes and the chosen proniosomal formula are presented in fig. 7. The DTA thermogram of clopidogrel showed a sharp endothermic peak at 179 °C which is corresponding to its melting point and indicated the crystallinity of clopidogrel [2]. Span 60, cholesterol and maltodextrin showed endothermic peaks at 54.4 °C, 144.2 °C and 294.9 °C corresponding to their transition temperatures, respectively [51, 52]. The plain proniosomes exhibited an appearance of a new endothermic peak at 191.1 °C. That may be explained on the basis of interaction of the proniosome components during the formation of the lipid bilayer [49]. The chosen proniosomal formula (F1) showed the shift of the endothermic peak of the lipid bilayer to 199 °C and disappearance of the endothermic peak of clopidogrel. These findings are in accordance with results reported by Patil (2015) [53]. These findings might suggest a change of clopidogrel structure from crystalline to amorphous state due to high entrapment efficiency of clopidogrel into proniosomes [54]. That may be the cause of the enhanced dissolution of clopidogrel from proniosomes because amorphous state contains no crystal lattice interactions or bonds that need to be broken [55].

**Effect of clopidogrel proniosomal formula (F1) on platelet aggregation**

The effect of the chosen clopidogrel proniosomal formula (F1) on platelet aggregation was measured as bleeding time and bleeding volume. Our results showed that clopidogrel proniosomal formula (F1) significantly (p ≤0.001) increased the bleeding time (682±1.4) min compared to both control group (1.99±0.27) min as well as clopidogrel group (3.32±0.33) min after 1 h of treatment. The results obtained after 3 h of treatment further confirmed the inhibitory effect of the proniosomal formula on platelet aggregation. The bleeding time in mice treated with F1 reached up to (9.86±1.70) min which was significantly (P<0.001) different from outcomes of control group (2.05±0.37) min and clopidogrel-treated group (5.11±0.54) min, (fig. 8A).

Moreover, the bleeding volume 1 h after treatment with clopidogrel proniosomal formula (F1) (0.22±0.019) ml was significantly (P<0.001) greater than control group (0.11±0.01) ml and clopidogrel group (0.17±0.02) ml. Similarly, after 3 h of treatment, the bleeding volume of mice treated with F1 (0.31±0.04) ml was significantly (P<0.001) higher than control group (0.15±0.02) ml and clopidogrel group (0.21±0.04) ml (fig. 8B).

**Effect of clopidogrel proniosomal formula (F1) on coagulation profile**

The effect of the chosen clopidogrel proniosomal formula (F1) on the coagulation profile was measured using coagulation markers such as prothrombin time and prothrombin activity as well as INR. The results of this study showed that clopidogrel as proniosomes was more potent compared to clopidogrel aqueous dispersion where 3 h post-treatment, clopidogrel proniosomal formula (F1) significantly (P<0.05) prolonged prothrombin time to (15.5±0.57) min and decreased prothrombin activity to (62.35%±5.28) and consequently increased the INR up to (1.52±0.12) compared to plain clopidogrel suspension that showed (14.17±0.36) min, (71.95%±1.76) and (1.33±0.05) for prothrombin time, prothrombin activity and INR respectively. The outcomes of the two groups were significantly (P<0.01) different from those obtained from a control group that showed prothrombin time, prothrombin activity and INR equal to (12.64±0.71 min, 82.64%±4.6, 1.15±0.07) respectively (fig. 9).

The above data agree with results reported by Karahulut et al.(2004)[56]. Moreover, from the above results, it is clear that the formulation of clopidogrel as proniosomes improved the antiplatelet effect of clopidogrel. That may be attributed to the enhancement of solubility and bioavailability of clopidogrel following formulation as proniosomes. These results are in accordance with Wen et al. (2011) [57] and Sengodan et al.(2009)[13] who reported that the prepared proniosomal formulation showed higher pharmacological effect than the plain drug. In addition, Ali et al.[2015] observed that liposomes of clopidogrel were more effective in the inhibition of platelet aggregation than orally administered clopidogrel [30]. Therefore, Proniosomes are emerging strategy for more effective drug delivery [58].

**In vivo study**

The In vivo behavior of the chosen proniosomal formulation (F1) was studied by measuring antiplatelet activity in adult male mice using platelet aggregation inhibition study and INR Measurement.

**Fig. 7: DTA thermogram of clopidogrel (a), Span 60 (b), cholesterol (c), maltodextrin (d), plain proniosomes (e) and the chosen clopidogrel proniosomal formula (f)**

**Fig. 8: Effect of clopidogrel proniosomal formula (F1) on platelet aggregation**

Values of test group are compared with control group: a (p ≤0.05), aa (p ≤0.01), aaa (p ≤0.001) and compared with Clopidogrel group: b (p ≤0.05), bb (p ≤0.01), bbb (p ≤0.001). Data are expressed as mean±SD, n=5
CONCLUSION
The present study deals with developing clopidogrel proniosomes. Clopidogrel-based proniosomes were successfully prepared as free-flowing powders with high entrainment efficiency in the range of 83.04±1.99 to 90.14±0.30. The proniosomal formulations showed higher dissolution profile than the plain drug. F1 (clopidogrel proniosomes prepared with cholesterol and Span 60 in 40:210 molar ratio) showed the highest in vitro release over other proniosomal formulations. F1 was found to be free-flowing powder of good flowability that is higher than that of maltodextrin powder. The niosomal vesicles formed by hydration of F1 proniosomes were spherical in shape with mean vesicle size of 217.9 nm with zeta potential value (+17.65 mv). FTIR study confirmed the absence of any chemical interactions between clopidogrel and other excipients. DTA study showed a change of clopidogrel structure from crystalline to an amorphous state. In vivo test showed that clopidogrel proniosomal formula enhanced the antiplatelet activity of clopidogrel. This approach could be promising in enhancing the dissolution, bioavailability, and efficacy of clopidogrel as well as to decrease its adverse effects.

AUTHORS CONTRIBUTIONS
All authors have contributed equally

CONFLICT OF INTERESTS
All authors have declared no conflict of interest

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