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Preparation and characterization of furosemide nanosuspensions

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ABSTRACT

Furosemide, a widely used loop diuretic, has a low aqueous solubility, and low permeability. Nanosuspensions have been widely used to increase the solubility of poorly soluble drugs. The aim of this study was to develop and characterize furosemide containing nanosuspensions. Furosemide nanosuspensions with Tween 80, were prepared using high pressure homogenization method using ultrasonic probe or ultra turrax, ball milling method, and combination of these methods. The physicochemical properties of furosemide, physical mixture and nanosuspensions were evaluated by FT-IR, DSC and X-ray analyses. Particle size, polydispersity index, zeta potential, solubility and permeability of nanosuspensions were also determined. FT-IR analysis revealed that characteristic peaks of furosemide were seen in all formulations. X-ray analysis indicated that crystalline structure of furosemide was preserved in nanosuspensions. The particle size of furosemide decreased significantly ($p < 0.05$) by using nanosuspension technology. Furosemide solubility was pH-dependent, and impact of nanosuspension on the solubility was more pronounced at lower pH values (e.g. pH 1.2). Furosemide permeability seemed to be influenced by nanosuspension preparation method. In conclusion, nanosuspension technology seems to be a promising approach for enhancement of solubility and permeability properties of poorly water soluble compounds, and it has an excellent potential to improve the bioavailability of such compounds.

1. Introduction

New active pharmaceutical ingredients generally have poor aqueous solubility and dissolution rate, and hence poor bioavailability [1–3]. It is estimated that more than fourty percent of compounds are poorly water soluble. Therefore, improving solubility and dissolution rate of poorly water soluble drugs are very important. However, it is difficult to develop drug products containing poorly water soluble compounds by conventional methods. Many approaches have been employed to enhance oral bioavailability of poorly soluble drugs such as addition of cosolvents, salt formation, adjusting pH [4], complexation with cyclodextrins [5], emulsions, micellar dispersions, solid dispersions, particle size reduction [6], and hydrotropy [7]. Also, nanosuspensions are widely used to increase solubility, dissolution and oral bioavailability of large number of poorly soluble drugs [8]. Nanosuspensions are submicron colloidal dispersions comprised of drug nanocrystals, stabilizing agents (e.g.surfactants and/or polymeric stabilizers), and a liquid dispersion medium [9]. The dispersion media can be water, aqueous solutions, or nonaqueous media [10]. By using nanosuspension technology, the particle size of drug crystals can be reduced that leads to an increase in the total surface area of the drug crystals. The dissolution rate of the poorly soluble drug is proportional to the surface area. As the particle size of the drug is reduced in nanosuspensions, surface free energy is increased with an increase in the surface area leading to potential particle aggregation or crystal growth. However, the presence of stabilizers helps to keep the dispersions physically stable and prevents reaggregation of the drug particles during the preparation process and shelf-life [3]. Various methods are used for the preparation of nanosuspension formulations; bottom up which is a nanoprecipitation, top down which is a homogenization and milling (both methods are utilized together), and spray drying methods [11]. Top down method which is a disintegration method helps to produce nanometer-sized particles of poorly water soluble drugs, and generally results in agglomeration and crystal growth. Therefore, stabilizers are usually used to prevent the crystal growth in this method [12]. A common approach for stabilization is electrostatic technique and steric prevention. This is achieved by adsorbing polymers onto to the drug particle surface and leads to electrostatic or steric stabilization by adsorbing molecules [13]. The stabilizers can be polymers including hydroxypropyl methylcellulose (HPMC), polyvinyl pyrrolidone (PVP K30) and surfactants including nonionic polysorbate (Tween 80) and ionic sodium lauryl sulphate (SLS). Nanosuspensions should contain stabilizing agents to inhibit

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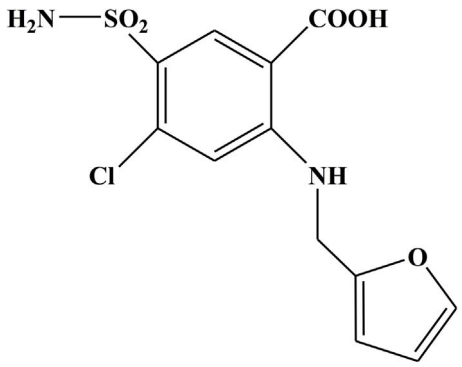

Fig. 1. Chemical structure of furosemide.

crystal growth [14]. Eventually, the solubility, dissolution profile and thus the bioavailability of the drug are improved by using nanosuspension technology. For this purpose, Tween 80 was used as a stabilizer for preparing furosemide nanosuspensions in this study. The drug was mixed with the stabilizer and homogenized with different instruments.

There are several advantages of nanosuspensions, such as, enhanced oral bioavailability, applicability to most drugs, reduced food effects, ease of preparation, and possibility of sterile filtration due to decreased particle size range [15].

Furosemide, 5-(aminosulphonyl)-4-chloro-2-[(2-fuanyl-methyl) amino] benzoic acid, is a loop diuretic drug widely used for the treatment of edema associated with heart disease, liver disease, renal disease including nephrotic syndrome (Fig. 1) [16,17]. Furosemide, which is a weak acid (acidic pK_a 3.8), is practically insoluble in water and its aqueous solubility increases as a function of pH. It is fairly rapidly absorbed from the gastrointestinal (GI) tract. Absorption following oral administration is influenced by the dosage forms [18]. According to the Biopharmaceutics Classification System (BCS), furosemide is categorized as a Class IV compound with poor water solubility and poor permeability [19]. Following oral administration, furosemide exhibits flip–flop pharmacokinetics [20].

The primary objective of this study was to improve the solubility and (hence permeability) of furosemide by preparing its nanosuspension formulation using homogenization and ball milling techniques. Tween 80 was selected as the stabilizer to minimize the surface free energy of the particles by confering a steric repulsion. Particle size and zeta potential values of raw furosemide, physical mixture and nanosuspensions were determined before and after lyophilisation. The physicochemical characteristics of raw furosemide, Tween 80, physical mixture and nanosuspension formulations were determined by means of FT-IR, DSC and X-ray analyses. Apical to basolateral permeability of raw furosemide, physical mixture and nanosuspensions were determined across Caco-2 cell monolayers 21 days after seeding.

2. Materials and methods

2.1. Materials

Furosemide was a generous gift from Sanofi- Aventis (Turkey). Polysorbate (Tween) 80 was purchased from Merck (Germany). Deionized water was used as the dispersion medium for preparation of furosemide containing nanosuspensions. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of nanosuspensions

Different methods were used either alone or in combination for preparation of furosemide nanosuspensions.

2.2.1.1. High speed homogenization (ultrasonic) method (UP). A suspension of furosemide with Tween 80 (0.5:1 (w/w)) was prepared in distilled water to a final concentration of 3% (w/v). The suspension was initially mixed by a magnetic stirrer for 15 min, and then mixed by an Ultrasonic Probe at 20% power for 1 min. The dispersion medium was removed by lyophilization (−55 °C, 0.01 mm Hg) for 72 h.

2.2.1.2. High speed homogenization (mechanical) method (UT). A suspension of furosemide with Tween 80 (0.5:1 (w/w)) was prepared in distilled water to a final concentration of 3% (w/v). The suspension was initially mixed by a magnetic stirrer for 15 min, and then followed by an Ultra Turrax at 11000 rpm for 3 min. The dispersion medium was removed by lyophilization for 72 h.

2.2.1.3. Ball milling method (BM). A suspension of furosemide with Tween 80 (0.5:1 (w/w)) was prepared in distilled water to a final concentration of 3% (w/v). The suspension was mixed by a magnetic stirrer for 15 min, and then ball milled at 200 rpm for 15 min. The dispersion medium was removed by lyophilization for 72 h.

2.2.1.4. Combination of high speed homogenization (ultrasonic) method (UP) and ball milling method (BM). A suspension of furosemide with Tween 80 (0.5:1 (w/w)) was prepared in distilled water to a final concentration of 3% (w/v). The suspension was mixed by a magnetic stirrer for 15 min, followed by an Ultrasonic Probe at 20% power for 1 min, and then ball milled at 200 rpm for 15 min. Finally, the dispersion medium was removed by lyophilization for 72 h.

2.2.1.5. Combination of high speed homogenization (mechanical) method (UT) and ball milling method (BM). A suspension of furosemide with Tween 80 (0.5:1 (w/w)) was prepared in distilled water to a final concentration of 3% (w/v). The suspension was mixed by a magnetic stirrer for 15 min, followed by an Ultra Turrax at 11000 rpm for 3 min, and then ball milled at 200 rpm for 15 min. Finally, the dispersion medium was removed by lyophilization for 72 h.

2.2.1.6. Physical mixture (PM). A physical mixture of furosemide with Tween 80 (0.5:1 (w/w)) was prepared manually using a mortar and pestle.

2.2.2. Saturation solubility of furosemide, physical mixture and nanosuspensions

The saturation solubility of furosemide, physical mixture and nanosuspenisons were determined at pH 1.2, 4.6, 6.8, 7.4. An excess amount of all samples was added to a suitable buffer, and then shaken continuously for 24 h in a water bath maintained at 50 rpm and 37 °C. After equilibration, samples were filtered through membrane filter (0.45 μm), and then analyzed at 277 nm wavelength using a Shimadzu UV/vis spectrophotometer. The solubility concentrations for each formulations were calculated using the calibration curves constructed for all different pH buffers.

2.2.3. Characterization of nanosuspensions

2.2.3.1. FT-IR spectroscopy. Infrared spectra of furosemide in powder, physical mixture and nanosuspensions were detected over the range of 4000–650 cm^{-1} with a Fourier transform infrared spectrometer (Perkin- Emler, USA).

2.2.3.2. X-ray diffractometer analysis. Ultima X-ray diffractometer (Ultima X-ray diffractometer, Tokyo, Japan) was used to determine the X-ray diffractograms of raw furosemide, physical mixture and nanosuspensions. The standard runs were performed at 40 kV voltage and a scanning rate of 0.02/min over a 2θ range of 2-40°.

2.2.3.3. Differential scanning calorimetry (DSC). A DSC Q 100 system (TA Instruments, Delaware, USA) was used to determine the thermal properties of raw furosemide, physical mixture and nanosuspensions.

Samples (5–20 mg) were sealed into an aluminum pan, and heated at a rate of 10 °C/min (10–200 °C) under nitrogen purge (50 mL/min). The changes in sample heat were monitored with respect to change in temperature. Empty aluminum pans were used as reference.

2.2.4. Particle size analysis and zeta potential measurement

The mean particle size, polydispersity index and zeta potential values of physical mixture and nanosuspensions were determined by Photon Correlation Spectroscopy (PCS) with a Zetasizer Nano ZS (Malvern Instruments, UK). Prior to measurements, each sample (10 mg) was diluted with distilled water (up to 10 mL), and then vortexed for 1 min. As the particle size of raw furosemide could not be measured by Zetasizer Nano ZS due to sensitivity of the instrument, it was measured by Malvern Master Sizer 2000. To evaluate the effect of lyophilization, particle size, polydispersity index, and zeta potential values of formulations were measured before and after the lyophilization. Three measurements were performed for each sample.

2.2.5. Permeability studies

Caco-2 cell monolayer was used to investigate the permeability of raw furosemide, physical mixture and nanosuspensions. Cells (passage number 28–32) seeded on 12-well plates (THIN CERTS, pore diameter 1 μm surface 1.13 cm^2) at a density of 30.000 cells/well were grown for 21 days. Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 50 unit/mL penicillin and 50 μg/mL streptomycin was used as the growth medium, and changed every other day until the permeability experiments. Before the experiment, transepithelial electrical resistance (TEER) values were measured and Caco-2 cells with TEER values > 600 Ω cm^{-2} were used for transport studies. Test compound (raw furosemide, physical mixture and nanosuspensions) were prepared in Hank's buffered salt solution (HBSS) containing 1% DMSO at a concentration of 20 μM, and added to the apical compartment (0.5 mL). Basolateral side was added only the control transport buffer containing 1% DMSO (1 mL). Samples were removed from the basolateral compartment 2 h after the incubation (37 °C and 30 rpm), and analyzed at 277 nm using a Shimadzu UV/vis spectrophotometer. All experiments were performed in triplicate. The permeability (P_{app}, cm/s) values were calculated as follows:

P_{app} = Rate of transport/(surface area x initial donor concentration)

2.2.6. Statistical analysis

All reported data were given as mean ± standart deviation (SD). Significance of the difference between particles sizes, solubility and permeability of formulations were evaluated using two way ANOVA test at the probability level of 0.05.

3. Results and discussion

In the literature, there are two studies available reporting the preparation of furosemide nanosuspensions [21,22]. The main difference between these studies and our study is the preparation method used for the furosemide nanosuspensions. The nanosuspensions in these studies were prepared by nanoprecipitation with sonication using dimethyl sulfoxide as a solvent and water as an antisolvent, and polyvinyl acetate as the stabilizer. We prepared nanosuspensions using high pressure homogenization method using ultrasonic probe or ultra turrax, ball milling method, and combination of these methods in our study. Compare to the other studies, our method is simple and more economical because fewer steps are required to prepare the nanosuspensions. Also, it is a safe method as only water is used for the preparation, so there is no risk of toxicity due to organic solvent residues.

3.1. Saturation solubility of furosemide, physical mixture and nanosuspensions

Bioavailability is defined to as the rate and extent of drug that is absorbed and becomes available at the site of drug absorption [23]. Bioavailability of a compound is affected by two main parameters namely solubility in the GI tract and permeability across the biological membranes [24]. For orally administered drugs, particle size may have an important effect on the bioavailability. By decreasing particle size and surface area, solubility increases and this leads to an increase in the bioavailability. Permeability across cell mebranes, and hence bioavailability, may be enhanced using of surfactants or polymers in nanosuspensions [25]. In this study, furosemide was selected as the model compound because of its low solubility and low permeability characteristics. Therefore, impact of its nanosuspension formulations on solubility and also permeability of furosemide can be evaluated.

The saturation solubilities of raw furosemide, physical mixture and nanosuspensions were displayed as a function of pH (pH 1.2, 4.6, 6.8, 7.4) in Fig. 2. The results of the solubility studies indicated that raw furosemide has a pH dependent solubility. The lowest solubility was obtained at pH 1.2 and the highest at pH 7.4 for raw furosemide (e.g. 1.2 μg/mL at pH 1.2, 14.32 μg/mL at pH 4.6, 16.3 μg/mL at pH 6.8, 19.03 μg/mL at pH 7.4). This observation is in agreement with the literature (e.g. 0.18 mg/mL at pH 2.3 and 13.36 mg/mL at pH 10.0) [26]. Unlike raw furosemide, variable solubility results were obtained for nanosuspension formulations. It is interesting to note that at pH 1.2, furosemide solubility was significantly increased by all nanosuspension formulations (29–45-fold increase when compared to raw furosemide; $p < 0.05$) except UP + BM method (1.2 fold increase). At pH 4.6, solubility of furosemide was increased 1.01–2.7 fold in

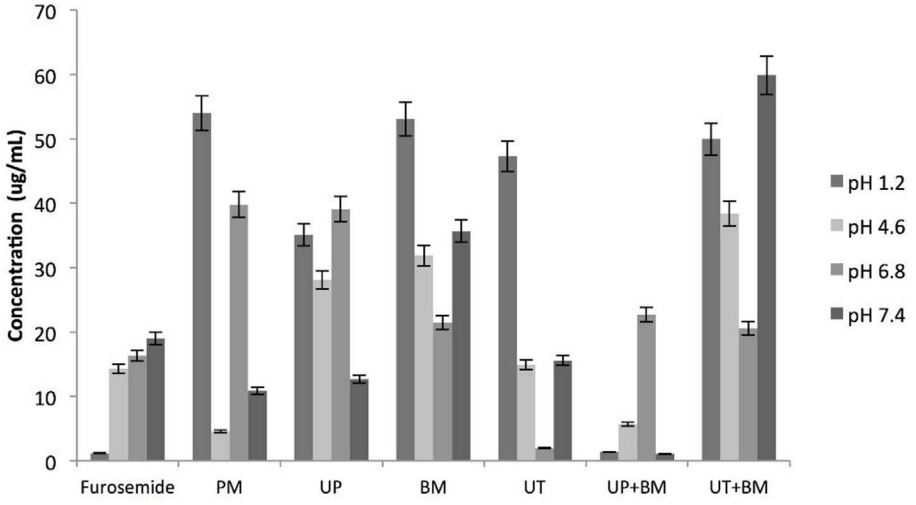

Fig. 2. Saturation solubility results of raw furosemide, physical mixture (PM) and nanosuspensions (mean ± SD; n = 3).

nanosuspension formulations prepared by UP, BM, UT, UT + BM methods, while solubility decreased in PM (3.5 fold decrease), and nanosuspension prepared by UP + BM method (2.5 fold decrease). Similarly, furosemide solubility was increased 1.3–2.4 fold for all nanosuspension formulations except UT method (about 80% decrease in solubility). In the case of pH 7.4, furosemide solubility was increased 1.9–3.1 fold for nanosuspensios prepared by BM and UT + BM methods only, for other methods, furosemide solubility was decreased by 45–95% of raw furosemide.

Collectively, all these results indicate that the impact of nanosuspension on the solubility will be more pronounced at lower pH values (e.g. pH 1.2), and also furosemide solubility appears to be affected at various levels by the method of nanosuspension preparation. Increase in solubility due to particle size reduction by preparing nanosuspensions with a suitable method can be expected to enhance dissolution rate and bioavailability.

3.2. Characterization of nanosuspensions

3.2.1. FT-IR spectroscopy

FT-IR spectra (4000-650 cm^{-1}) of raw furosemide, Tween 80, physical mixture and nanosuspensions are displayed in Fig. 3. Similar to the literature, the spectrum of furosemide displayed characteristic

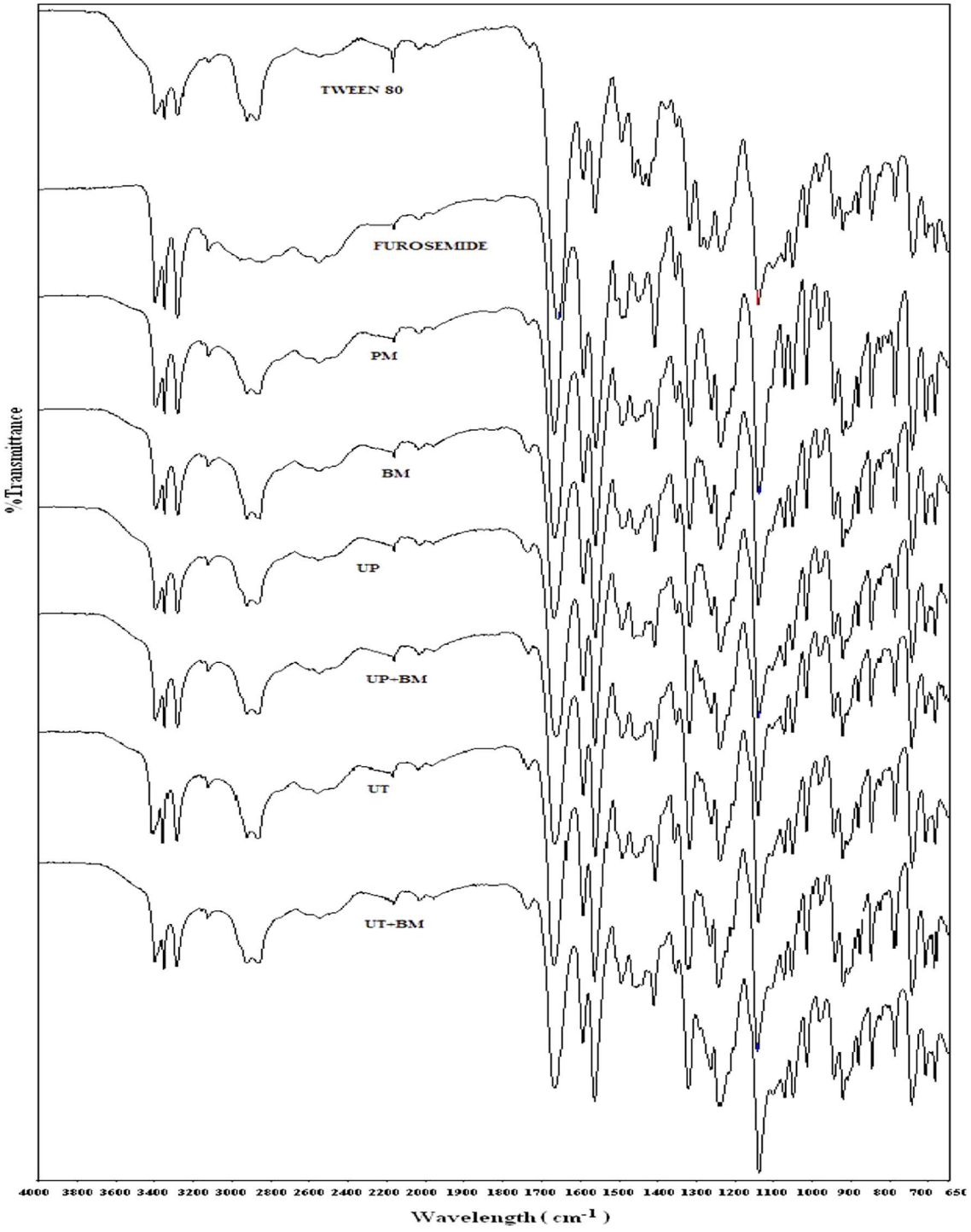

Fig. 3. FT-IR results of Tween 80, raw furosemide, physical mixture (PM) and nanosuspensions.

peaks at 1136 cm^{-1} (symmetric SO_2), 1315 cm^{-1} (asymmetric SO_2), 3397 cm^{-1} (asymmetric N-H), 3349 cm^{-1} (symmetric N-H), 1666 cm^{-1} (C=O), 3123 cm^{-1} (aromatic C-H), 742 cm^{-1} (C-Cl), 1559 cm^{-1} (furan ring and C-H stretching vibration band), 1590 cm^{-1} (C=C), 1260 cm^{-1} (carboxylic acid, C-O), 1237 cm^{-1} (Furan ring C-O-C) [16,27,28]. FT-IR analysis results showed that chemical structure (characteristic peaks) of furosemide was preserved in all nanosuspensions indicating that preparation method had no effect on the stability of furosemide.

3.2.2. X-ray diffractometer analysis

X-ray analysis is used to determine the crystalline structure of a drug. Compare to amorphous form, crystalline forms are physically more stable [29]. The X-ray diffraction patterns of the raw furosemide, Tween 80, physical mixture and selected nanosuspensions are given in Fig. 4. Physical mixture and Tween 80 are used as controls. Nanosuspensions prepared by ball milling method, and combination of high speed homogenization (mechanical) and ball milling methods (UT + BM) were selected according to saturation solubility results for X-ray analysis. Tween 80 has an amorphous structure whereas furosemide has a crystalline structure. Although characteristic furosemide diffraction peaks were preserved in nanosuspensions, the intensity of the peaks were lower than that of raw furosemide, probably due to interaction between excipients and furosemide, and also seemed to be influenced by the preparation method of nanosuspensions. These X-ray results showed that crystalline state of furosemide was preserved in the nanosuspensions after milling and homogenization processes [30] indicating that stable and more soluble furosemide nanosuspensions were prepared in our study. Similar observations were also reported for other compounds in the literature [30–32].

3.2.3. Differential scanning calorimetry (DSC)

DSC analysis gives information about the thermal properties of a formulation, and the physicochemical state of drug in the formulation. DSC analysis was performed to investigate the effect of excipients and preparation method on furosemide. The DSC thermograms of furosemide, Tween 80, physical mixture and selected nanosuspensions (prepared by BM and UT + BM methods) are shown in Fig. 5. The DSC thermograms of furosemide and Tween 80 showed a melting endothermic peak at 210 °C, −20.55 °C respectively. Our DSC results in regard to furosemide and Tween 80 agree well with the literature [33,34]. On the other hand, the melting peak of furosemide was absent in the DSC thermogram of nanosuspensions prepared by BM and UT + BM methods indicating that furosemide was covered with surfactant in the nanosuspension.

3.3. Particle size analysis and zeta potential measurement

The primary goal was to have a reduction in particle size of nanosuspensions. The average particle size, polydispersity index and zeta-potential values were measured immediately after the preparation of the nanosuspensions and after freeze-drying of the nanosuspensions (Table 1). Particle size of raw furosemide (9759 ± 1010 nm) was significantly higher than those of physical mixture and all nanosuspension formulations ($p < 0.05$). Similar particle size values were obtained before and after freeze-drying for nanosuspension formulations prepared by PM, BM, UT and UT + BM methods with no significant difference between them ($p > 0.05$). On the other hand, particle size was significantly increased after freeze-drying for nanosuspensions prepared by UP and UP + BM methods ($p < 0.05$). This observation could be attributed to the aggregation of the particles after freeze-drying. Similar observations were also reported in the literature supporting our results [35–37].

Polydispersity index (PDI) value is used to evaluate the particle size distribution. If the value of PDI is below 0.5, it indicates that particle size distribution is narrow. PDI values of all nanosuspension

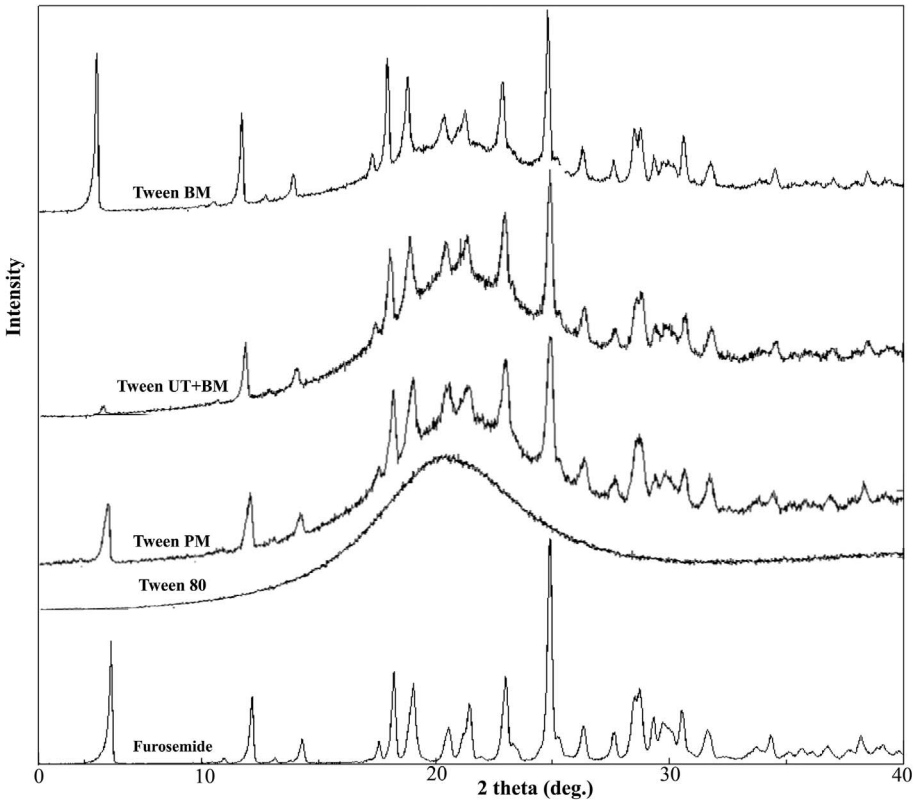

Fig. 4. X-ray diffractograms of ball milling (BM) combination of high speed homogenization (Mechanical) method (UT) and ball milling (BM), physical mixture (PM), furosemide, Tween 80.

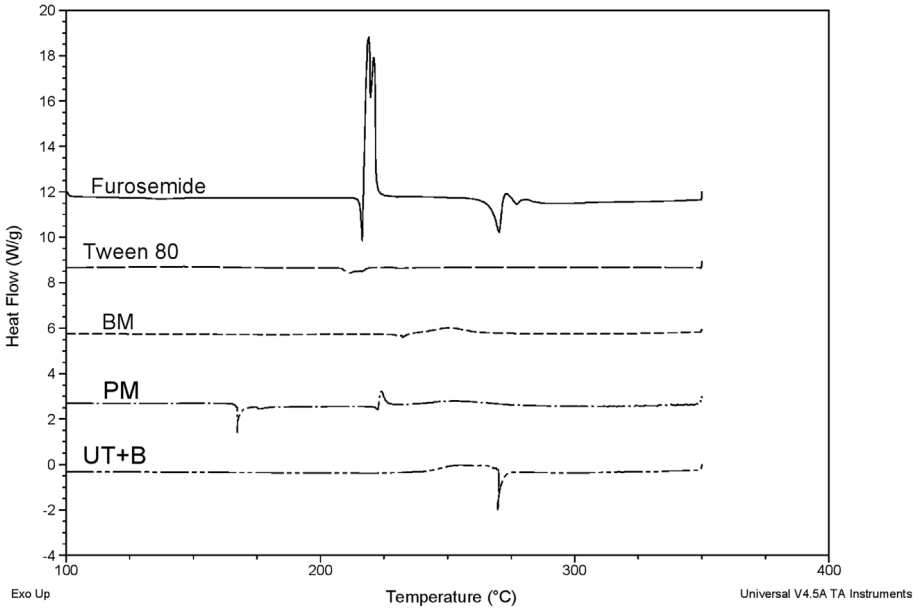

Fig. 5. Differential scanning calorimetry thermograms of the furosemide, Tween 80, physical mixture (PM), ball milling (BM), combination of high speed homogenization (Mechanical) method (UT) and ball milling (BM).

Table 1
Mean particle size (nm), polydispersity index and zeta potential values of nanosuspensions before and after lyophilization (mean ± SD: n = 3).

Methods	Mean particle size		Polydispersity index		Zeta Potential	
	Before	After	Before	After	Before	After
PM	2814 ± 24.0	2762 ± 57.1	0.422 ± 0.082	0.311 ± 0.064	−12.0 ± 0.3	−29.1 ± 0.6
BM	2658 ± 97.1	3145 ± 129.7	0.182 ± 0.019	0.248 ± 0.081	−13.2 ± 1.4	−30 ± 0.2
UP	1621 ± 76.7	3625 ± 272.2	0.247 ± 0.083	0.320 ± 0.054	−8.71 ± 2.0	−29.9 ± 1.0
UP + BM	1467 ± 52.8	2830 ± 161.8	0.258 ± 0.094	0.171 ± 0.127	−8.31 ± 1.6	−30 ± 0.5
UT	2982 ± 1035	2661 ± 283	0.11 ± 0.03	0.353 ± 0.072	−13,6 ± 1.2	−35.9 ± 0.4
UT + BM	2220 ± 997.3	2573 ± 69.9	0.168 ± 0.12	0.189 ± 0.041	−10.8 ± 1.1	−33.7 ± 0.7

formulations are less than 0.5 confirming narrow particle size distributions (Table 1).

The zeta potantial is a measure of the electric charge at the surface of the particles indicating the physical stability of colloidal systems. It is recommended that zeta potential values between −10 and + 10 mV are considered approximately neutral, while zeta potentials greater than +30 mV or less than −30 mV are considered strongly stable [38]. In this study, Tween 80, a non-ionic surfactant, was used as a stabilizer which provides electrostatic stabilization [39]. Both before and after freeze-drying, zeta potential values of all nanosuspensions were negative charge (Table 1) indicating that all nanosuspension formulations are stable colloids.

3.4. Permeability studies

According to BCS, furosemide is classified as a low permeability compound [40]. In literature, furosemide permeability across Caco-2 cells was reported to be in between 0.086 -10 × 10^{-6} cm/s [41–45]. Yamashita et al. reported that the apical to basolateral permeability of furosemide across Caco-2 monolayer was pH dependent (11 ± 0.01 × 10^{-6} cm/s at pH 6.0, and 0.045 ± 0.004 × 10^{-6} cm/s at pH 7.4 [41]. Furthermore, compounds can be classified according to permeability values as poorly (0–20%; P_{app} < 1 × 10^{-6} cm/s), moderately (20–70%; P_{app} between 1−10 × 10^{-6} cm/c) and well (70–100%; P_{app} > 10 × 10^{-6} cm/s) absorbed compounds [46]. Similar to literature, permeability of raw furosemide across Caco-2 cells was low (P_{app} = 9.6 × 10^{-6} cm/s; Fig. 6). However, permeabilities of furosemide nanosuspensions were either similar (for BM, UT + BM, UP + BM methods; p > 0.05) to raw furosemide, or significantly higher (for PM, UT, UP methods; p < 0.05) than raw furosemide (Fig. 6). These results clearly indicate that permeability and hence bioavailability of furosemide can be significantly increased using a suitable nanosuspension preparation method. Also, furosemide seemed to be a moderately absorbed based on its permeability values.

4. Conclusion

In this study, three different methods were used either alone (high speed homogenization (Ultrasonic; UP), high speed homogenization (Mechanical; UT), and ball milling (BM methods) or in combination (UP + BM, UT + BM) for preparation of furosemide nanosuspensions. Tween 80 was used as a stabilizer for preparation of all nanosuspensions. Physicochemical properties of raw furosemide, physical mixture and nanosuspensions were evaluated by FT-IR, DSC and X-ray analyses. The results obtained from these studies clearly demonstrated that nanosuspension formulations were prepared successfully. Particle size, polydispersity index, zeta potential, pH dependent solubility and apical-to-basolateral permeability of nanosuspensions were also determined. The results obtained from this study indicated that furosemide solubility and permeability were increased by preparing its nanosuspensions. Although the average particle size of the nanosuspension formulations was significantly decreased as compared to raw furosemide and physical mixture, increase in solubility was more pronounced at lower pH values (e.g. pH 1.2) only. On the other hand, furosemide permeability was dependent on nanosuspension preparation method. It was either similar (for BM, UT + BM, UP + BM methods) or significantly higher

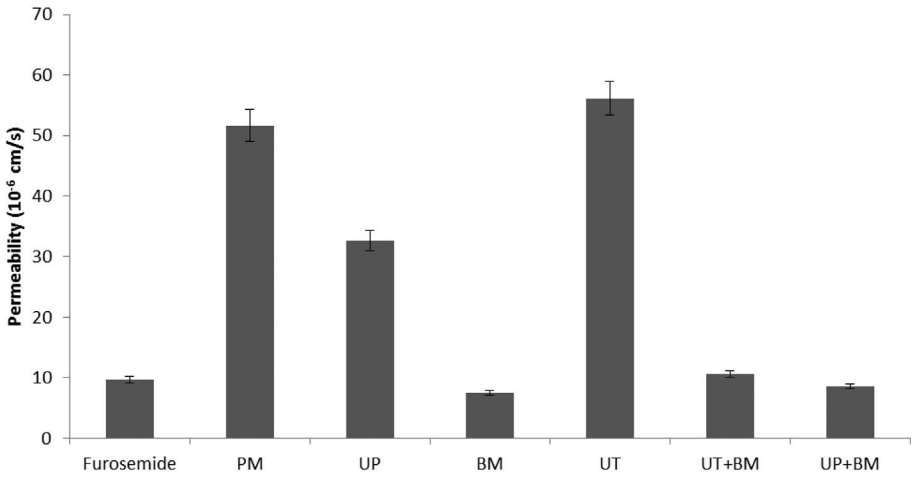

Fig. 6. Permeability values of raw furosemide, physical mixture (PM) and nanosuspensions across Caco-2 monolayers.

(for PM, UT, UP methods) than raw furosemide. In conclusion, nanosuspension technology can be used to enhance the solubility and permeability properties of a poorly water soluble compound, and it has an excellent potential to improve the bioavailability of such compounds.

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Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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