Research Article

Applying Nanotechnology to improve the Bioavailability of Qurcetine

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Abstract
Many pharmaceutical agents possess low solubility and dissolution behavior. This drawback limited their applications in the pharmaceutical field. The aim of this study is to apply nanotechnology to improve the physicochemical properties of pharmaceutical agents so as to improve its bioavailability and, hence, its therapeutic and clinical activity. Nanoprecipitation technique was optimized in order to obtain nanoparticles particles of Quercetin with enhanced solubility and dissolution rate properties. In order to attain this target, there are Preparation and physicochemical characterization of Quercetin nanoparticles, where nanoprecipitation method was applied to prepare the nanoparticles and different parameters were studied to obtain optimum nanoparticles of the drug including, Effect of polymer type , Effect of polymer concentration, Effect of homogenization time and Effect of homogenization intensity. On the Pharmacokinetic evaluation of the prepared Quercetin Nanoparticles , the optimum drug nanoparticles, as well as, the untreated drug was given to rabbits orally in a dose equivalent to 10 mg/kg. Different parameters were studied including, The maximum concentration Cmax, The time of maximum ,concentration Tmax ,The area under plasma concentration-time curve AUC, The area under first moment curve AUMC,The elimination rate constant ,The half-life elimination, The absorption rate constant and The half-life absorption, Results showed that the prepared nanoparticles produced significant increase in the AUC0-∞ (from 113.4 to 341.2 ng/ml.hr) suggesting increasing the bioavailability of the drug. This is accompanied with increasing Cmax from (25.2 to 60.4 ng/ml) and slight decrease in the Tmax value to 1.6 hr.

Keywords: Quercetin, Nanotechnology, pharmaceutical

Introduction
Nanotechnology can simply be defined as the technology at the scale of one-billionth of a metre. It is the design, characterization, synthesis and application of materials, structures, devices and systems by controlling shape and size at nanometer scale (Stylios; 2005, The Royal Society 2004). There are various methods used for preparation of Nano materials. Ionic gelation technique, relies on the comple-xation of the positive or electric charge of the hydrophilic compound along a multivalent cationic (e.g. calcium chloride) or polyanionic (e.g. Na tripoly-phosphate) to create extremely glutinous gel particle having a size range within the vary of a metric linear unit (Mudgil, 2012). Another technique, Nanoprecipitation Method In this method, the polymer is going to be precipitated from the organic solvent and the organic solvent diffuses in the hydrophilic medium with or without the help of a surfactant. (Nagavarma, 2012). In addition, Solvent Evaporation Techni-que In this technique, the volatile solvents are used to prepare polymeric solutions through emulsification. In the past, mostly chloroform and Dichloromethane were used for preparation of polymer solutions but currently ethyl acetate is used due to its better toxicological profile. (Nagavarma, 2012). Other methods are Dialysis method and Emulsification/ solvent diffusion. In dialysis method, dialysis tube is used with suitable molecular weight cut off and organic solvent carrying polymer is placed. With the help of this technique, narrowly distributed and

smaller sized nanoparticles could be prepared. (Nagavarma, 2012). Emulsification/ solvent diffusion This technique is the amended form of solvent evaporation technique. Here the polymer is mixed with partially hydrophilic solvents viz. (Nagavarma, 2012). So Nanoprecipitation Method was for the preparation of the nanoparticles in this thesis as this method is economic and requires relatively short time. In addition the availability of the equipment represents a very important advantage. For characterization of the prepared nanoparticles, Differential scanning calorimetry (DSC) was used that relies on the measurement of the difference between the heat flow vs. temperature relation of the sample and the heat flow vs. temperature relation of a standard. There are many types of calorimeters and the criteria for their classification. Pharmacokinetics is the study of kinetics of absorption, distribution, metabolism and excretion (ADME) of drugs and their corresponding pharmacologic, therapeutic, or toxic responses in man and animals'' (American Pharmaceutical Association, 1972). Applications of pharmacokinetics studies include bioavailability measurements, effects of physiological and pathological conditions on drug disposition and absorption, dosage adjustment of drugs in disease states, if and when necessary ,correlation of pharmacological responses with administered doses ,evaluation of drug interactions and clinical prediction using pharmacokinetic parameters to individualize the drug dosing regimen and thus provide the most effective drug therapy.

Methodology

Materials

- Quercetine (QRT) was purchased from from Sigma-Aldrich, Co. USA.
- Hydroxypropylmethylcellulose (HPMC) was obtained from Sigma-Aldrich, Co. USA.
- Sodium Dodecyl Sulfate (SDS) was obtained from Sigma-Aldrich, Co. USA
- All other chemicals, reagents and solutions were of analytical grade and used without purification.
- Reference standards of quercetin, Acetonitrile HPLC grade were purchased from sigma Aldrich, USA.
- Ethyl acetate were purchased from sigma-Aldrich, USA.
- All other reagents were of analytical grade.

Equipments

- HPLC: Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detectorRotaevaporator, and Magnetic stirrer, Heidolph, Germany.
- Thermostatically controlled shaker water bath, Polyscience®, USA.
- UV/VIS double beam spectrophotometer, Spectronic, Genesys, Milton Roy Co., USA.
- Infrared spectroscope: IR-470,Shimadzu Co., Japan.
- Differential scanning calorimeter: DSC-50, Shimadzu Co., Japan.
- X- Ray Diffractometer : Jeol, SPX 60-PA, Japan.
- Centrifuge (MPW-365, Mechanika, Precyzyjna, Poland)
- Electronic balance, Metler Co., Switzerland.
- ultrasonic probe sonicator (sonica, vibracell, USA)
- Ultrasonic water bath, Bransonic 220 Zurich, Germany.
- USP sieve set, Glenammer, Ayrshire, Scotland.
- Homogenizer, (Hielscher, Germany)
- Hot air oven.

experimental

Preparation of QRT nanoparticles

QRT suspensions were produced by antisolvent precipitation method under sonication. Briefly, Ethanol 99% against water was used as solvent and antisolvent in a ratio of 1:20 respectively. The organic solution of QRT was prepared by dissolving QRT in 10 mL Ethanol. The resulted solution was injected into 200 mL 0.15% (w/v) aqueous solution (containing HPMC and SDS, 2:1, w/w), cooled to below 8°C in an ice-water bath and kept under sonication condition.

The precipitation rate was controlled throughout the process by maintaining the temperature below 8°C using an ice-water bath. The particle size reduction was done with an ultrasonic probe sonicator (sonica, vibracell, USA) at ultrasonic power input of 300 W for 10 time length. The probe with a tip diameter of 10 mm was immersed 15 mm. The suspensions were kept under vacuum at room temperature for 2 h to remove the organic solvent. Then, the QRT suspensions were further homogenized by homogenization, using a homogenizer (Hielscher, Germany) for 8 minutes to obtain the final product. The homogenized suspension was evaluated for the particle size distribution.

Studying of different parameters affecting nanoparticles preparations

Effect of polymer type

Different polymers were employed to determine the most suitable one that produce the smallest particle size. The polymers used were Hydroxy-propylmethyl Cellulose (HPMC), Polyvinyl Pyrolidone (PVP K-30) and Polyethyleneglycol (PEG-4000, PEG-6000 and PEG-8000). The polymer that produced nanosized particles were subjected to further studies for the other parameter.

Effect of polymer concentration

Hydroxyl propyl methyl cellulose , that produced the nanosized particles (in section 2.1.) was prepared in different concentrations (f1, f4 and f7) in order to select the appropriate concentration succeeded in producing of the smallest particle size within the nano-scale.

Effect of sonication intensity

Hydroxyl propyl methyl cellulose in in selected concentration was subjected to studying the influence of sonication intensity. This parameter was studied at three levels (f_1,f_{10} and f_{11}).

Effect of homogenization intensity

The formula that produced the smallest particle size (in section 2.3.) was further studied for the effect of homogenization intensity were examined. The most suitable intensity was. f_1 : f13 (25%) subjected to further studies.

Effect of homogenization time

The formula that produced the smallest particle size was further studied for the effect of homogenization time. Three different times (f_1,f_3,f_{12} and f_{13}) were examined to give the best time for the smallest particle size within the nano-scale..

The different studied parameters were summarized in **table 1**.

Characterizations of the prepared nanoparticles Dynamic Light Scattering (DLS) Analysis and Zeta Potential Measurement

A liquid sample was diluted several times using Milli-Q water purifier and analyzed using DLS (Zetasizer Nano-ZS instrument, Worcestershire, United Kingdom). By placing samples in the cuvette, both particle sizes and the zeta potentials of the synthesized nanoparticles were determined. The samples were prepared for analysis at room temperature (25°C) and measured at 37°C in triplicates. Results were presented as mean ± SD (standard deviation).

determination of aqueous solubility

UV Spectrophotometric Analysis

Stock solution of QRT (one mg/ml) was prepared by dissolving the drug (25 mg) in the minimum amount of absolute ethanol. The volume was then completed to 25 ml using distilled water (stock I). In a 100 ml volumetric flask, 2 ml from stock I was transferred and diluted with distilled water to prepare 20 µg/ml (stock II). Appropriate volumes of stock II solution were transferred and diluted to 10ml with distilled water to obtain a concentration range of 2-18 µg/ml.

The absorption spectrum of the drug in each solution was scanned spectrophotometrically in the ultraviolet region (200–400 nm) against a suitable blank. The λ_{max} was determined with its corresponding absorbance. Each reading was the average of three determinations. Finally, standard calibration curve was constructed.

Solubility Studies

Solubility studies were carried out according to Higuchi and Connors reports. QRT or its equivalent as PM and nanoparticles were added in amounts beyond its solubility to 25 ml of phosphate buffer solution (pH 7.4) in stoppered flasks. The flasks were positioned in a shaker maintained at 25°C for 72 hours to reach equilibrium. The content of each flask was passed through 0.22µm filter unit and its

concentration was determined spectrophotometricaly by measurement of UV absorption at 254 nm against a suitable blank.

Powder X-Ray Diffraction (PXD)

The X-ray diffract grams of QRT, the polymers and selected prepared nanoparticles were obtained using X-Ray apparatus (Joel XR diffract meter). The source of radiation was CuKα radiation operated at 35 KV and a current of 15 mA. The diffract grams were obtained using continuous mode with 2θ values ranging from 4 to 100 at a rate of 2 degrees/min.

Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR spectroscopy was carried out using potassium bromide (KBr) method typically, 1–4 mg of each sample was mixed with KBr and compressed into discs. Scanning was carried out using NICOLET 6700 FT-IR spectrophotometer over the range of 600–4000 Cm−1. A blank KBr pellet was used as a reference.

Differential Scanning Calorimetry (DSC)

Thermal investigations of QRT, the polymers, and the selected nanoparticles were measured using differential scanning calorimetry (Perkin Elmer, 2-C, NY, USA). Procedures were done as previously described by several workers.5,13 The sample was heated from 30°C to 300C at a rate of 10°C/min.

HPLC analysis

Device Specification:

Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector.

Standard preparation:

Quercetine stock solution of 100 µg/ml in ethyl acetate was prepared, then serial dilution spiked on plasma to obtain conc. Of 250ng/ml, 100ng/ml, 75ng/ml, 50ng/ml, and 25ng/ml) then samples treated and reconstituted with 150 µl mobile phase, filtered using 0.22 µm syringe filter then 100µl was injected.

Sample preparation:

2 ml of Ethyl acetate were added to each of the14 samples (500 µl each), Vortexed for 2 min then centrifuged at 15,000 RPM for 10 min in cooling centrifuge then take the supernatant and evaporate under nitrogen stream then reconstitute sample with 150 µl mobile phase and then each of them were filtered using 0.22 µm syringe filter then 100 µl were injected.

HPLC analysis conditions:

- Column C18 thermo: 4.6x250mm, 5µm
- Mobile phase: Buffer (0.1 % phosphoric acid in water) and Acetonitrile
- Mode of elution: Isocratic
- Flow rate: 1ml/min
- Temperature: Ambient
- Wavelength: 254 nm

Pharmacokinetic Study

Animal

Among the tested samples, formula F6 was selected for the pharmacokinetic studies. This formula produced the smallest particle size The pharmacokinetic studies were conducted on healthy male New Zealand rabbits weighing 2.0–2.20 kg. Rats were housed in a temperature- and humidity-controlled environment (25°C, 65% RH) and maintained on a 12-hour light/dark cycle for 3 days before starting the experiment. In order to avoid the effect of food, animals were fasted overnight with free access to water.

The guidelines followed for the welfare of the animals are described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 39 All the experiments were also approved by the commission on the ethics of scientific research, faculty of pharmacy, Minia University .

Dosing and Sampling.

Animals are allocated into two groups, each consisting of 3 rabbits.

- The first group received untreated QRT in a dose level of 10 mg/kg.
- The second group received formula F6 in a dose equivalent to 10 mg/kg.

The doses were given orally and suspended in starch mucilage, as a single dose via feeding tubes. For blood sampling, a cannula was inserted into the marginal ear vein. Blood samples (2.5ml) were withdrawn periodically at specified time

interval into heparinized tubes. Once withdrawn, the samples were centrifuged at 5000 rpm and 4°C for 10 mins to separate plasma which is kept at −80°C pending analysis.

Pharmacokinetics Analysis
Data obtained from UHPLC experiments were utilized to establish plasma concentrations-time curve. In addition, PK solver program was used to calculate the pharmacokinetic parameters.40 All data are presented as mean ± SD.

Results and discussion
Studying of different parameters.

Effect of polymer type
Different polymers were examined for their abilities to form particles within the nano size by the nano precipitation method. These polymers were listed in table 1. Particles prepared with HPMC showed, relatively, the smallest particle size. Other polymers Showed particles of larger size which is not promising to prepare nanosized one, figure 1. QRT nanoparticles prepared with HPMC was selected for further studies in order to investigate the influence of different parameters on the particle size.

Effect of polymer concentration
It could be observed from table 1 that increasing the opolymer concentration resulted in decreasing the particle size. Figure 2 showed that increasing the polymer concentration from 50% to 200% resulted in a significant reduction in the particle size from 1763 nm to 1189. nm. With regard to Polydispersity Index (pdi), the reduction in the particle size was accompanied by a slight reduction in pdi value from 0.767 to 0.734.

Effect of sonication intensity
It could be observed from figure 3 that increasing sonication intensities resulted in reduction of particle size. For example, increasing the sonication intensity from 25% (formula F10) to 75% (formula F11) resulted in a significant reduction in the particle size of QRT from 1047 to 978.5nm. This reduction was also accompanied by a decrease in the pdi value which became 0.469, table 1.

Effect of homogenization time
It could be observed from figure 4 that increasing homogenization time resulted in reduction of particle size. For example, increasing the homogenization time from 15 min (formula F11) to 45 min (formula F13) resulted in a significant reduction in the particle size of QRT from 978.5 to 189 nm. This reduction was also accompanied by a decreases in the pdi value which became 0.518,table 2

Zeta potential studies (ζ).
zeta-potential occurs at the slipping plane outside the polymer layer. The magnitude of the "apparent" zeta-potential depends on the charge of the polymer layer and the thickness of that layer relative to the Debye length. For thick polymer layers, the potential derived from the underlying particle is masked, and the resulting zeta-potential is effectively the Donnan potential of the layer.

For thinner polymer layers, the "apparent" zeta potential is influenced by both the particle potential and the coating potential .Values of zeta-potential above ±30 mV were customarily considered moderately stable against aggregation due to charge stabilization, i.e.

the electrostatic repulsive forces are high enough to prevent aggregation. These "rules of thumb" discussed in more detail in Riddick (1968)22 were describe how to measure zeta-potential for engineered nano materials (ASTM E2865-12 and ISO 13099-1, -2, and -3),5–7 but provide minimal guidance on how to report those measurements. Additionally, these standards are not freely available, may not satisfy regulatory needs, and there is little standardization of measurement methods and reporting.

Physicochemical characterization of the prepared nanoparticles DSC
The DSC thermogram of QRT, figure 10, shows a small endothermic peak around 100°C corresponding to the release of water of hydration. Another characteristic sharp endo-thermic peak appeared at 325°C that correspond-ding to its melting point. Results were similar to that mentioned elsewhere (references). The DSC

thermogram of HPMC Showed no characteristic peaks. Regarding SDS, figure XC), the thermogram showed a small endothermic peak at 100°C due to water evaporation. In addition, a sharp characteristic peak appeared at 198°C corresponding to the melting point of the solid sample. The DSC thermogram of the prepared nanoparticles A, showed the dehydration peaks of QRT and SDS at their proper position around 100°C. the melting point endothermic peak appeared broadly at 300-350 °C suggesting the possibility of presence of an interaction.

With regard to the prepared nanoparticles B, the peaks were similar to that of A, but, with more brooding of the melting point characteristic peak of QRT. Suggesting the possibility of presence of interactions.

For the prepared nanoparticles C, D and E, the melting point characteristic peak of QRT was completely disappeared suggesting the presence of more pronounced interaction.

Results and discussion.

In order to estimate the plasma concentration of the treated samples, a calibration curve of standard QRT was constructed at λ_{max} of 254 and depicted in figure 13. from the figure it could be observed that the slope of the straight line was 307.463 with a regression coefficient r= 0.999 that could be considered satisfactory.

Table (1): Effect of different parameters used in the preparations of Quercetin nanoparticles.

Type of polymer		Concentration of polymer	Sonication intensity	Homogenizon time
F1	HPMC	50%	50%	5min
F2	HPMC	50%	50%	10min
F3	HPMC	50%	50%	15min
F4	HPMC	100%	50%	5min
F5	HPMC	100%	50%	10min
F6	HPMC	100%	50%	5min
F7	HPMC	200%	50%	5min
F8	HPMC	200%	50%	10min
F9	HPMC	200%	50%	15min
F10	HPMC	200%	25%	15min
F11	HPMC	200%	75%	15min
F12	HPMC	200%	75%	30min
F13	HPMC	200%	75%	45min

Table (2) : the average particle size and zeta potential for the prepared particles using HPMC

Formula		Average particle size(nm)	Range of particle size(nm)	Pdi	Z potential
F1	F1	4393	(900-8000)	0..307	
F2	F2	2298	(300-8000)	0.511	
F3	F3	2616	(900 – 8000)	0.435	
G1	F4	1763	(200-8000)	0.767	
G2	F5	1739	(300-8000)	0.493	
NA(G3)	F6	1566	(200-7000)	0.69	-30.4
H1	F7	1525	(200-8000)	0.778	
H2	F8	1289	(150-2000)	0.695	
H3	F9	1189.	(1000-2000)	0.734	
NB	F10	1047	(400-8000)	0.369	-33.4
NC	F11	978.5	(150-7500)	0.469	-36.3
ND	F12	901.2	(90-8000)	0.453	-35.1
NE	F13	189	(110-8000)	0.518	-36.4

Table(3): Pharmacokinetic parameters of untreated QRT and the nanoparticles F14.

	Units	QRT	Nanoparticles
A	ng/ml	1114.36	221.79
Ka	h^{-1}	0.625	0.738
Ke	h^{-1}	0.587	0.545
Cmax	Ng/ml	25.28	60.455
Tmax	H	1.9	1.6
AUC 0-∞	ng/ml.h	113.411	341.219
AUMC0-∞	$ng/ml.h^2$	374.329	1449.63
MRT	H	3.300	4.24

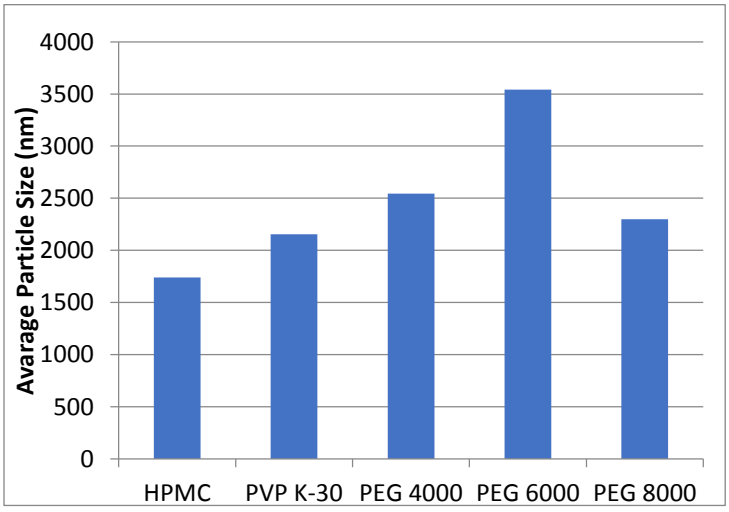


Figure (1): effect of different polymers on particle size

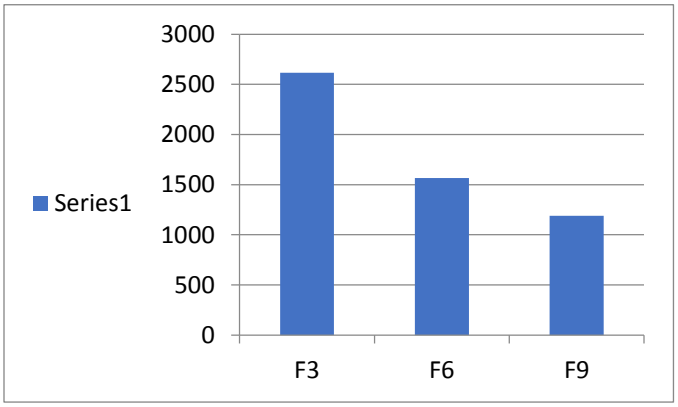


Figure (2): Effect of polymer concentration

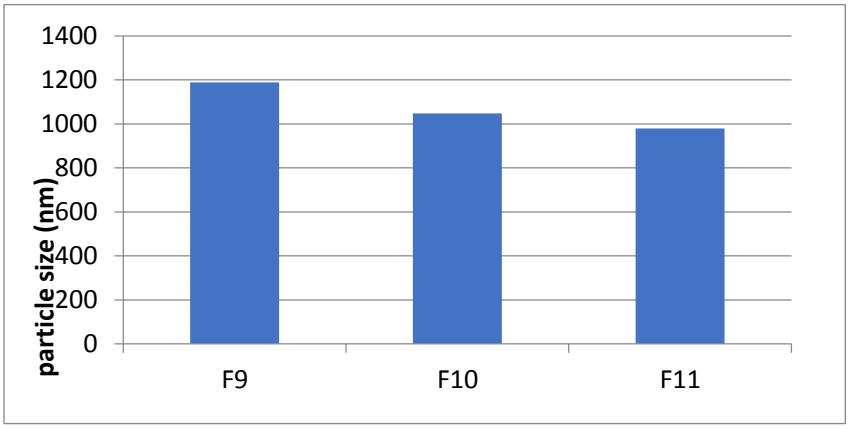


Figure (3) : Effect of sonication intensity on particle size

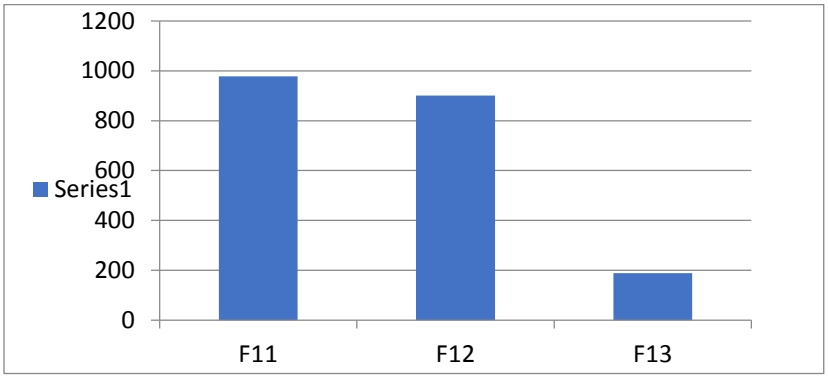
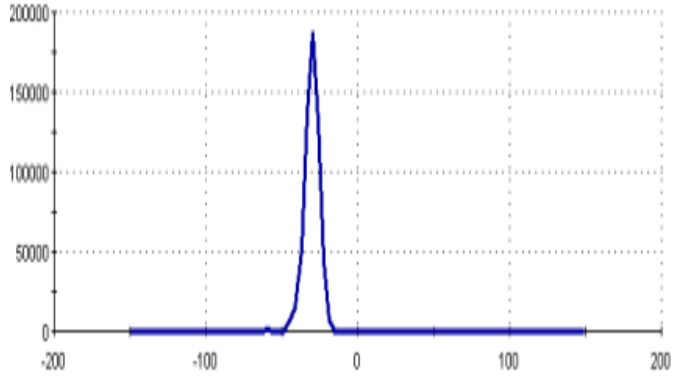


Figure (4): Effect of homogenization time on particle size



Figurer (5): zeta potential of formulation F6

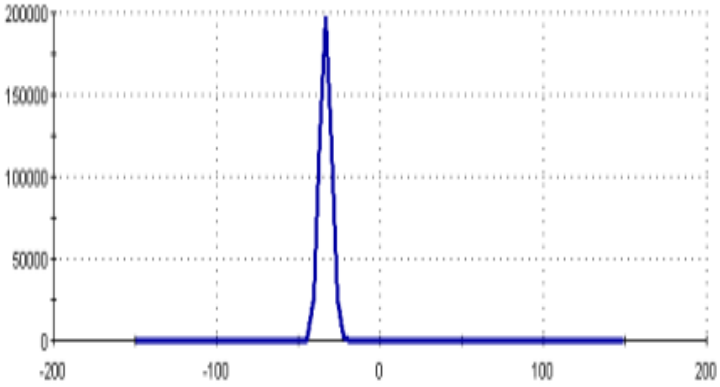


Figure (6): zeta potential of formulation F10

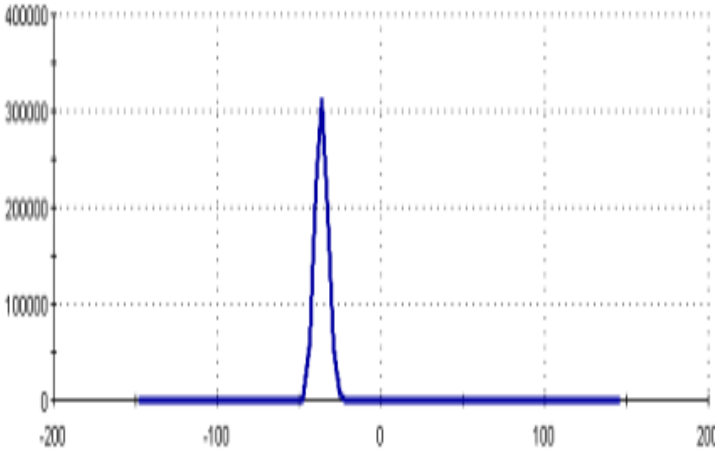


Figure (7): zeta potential of formulation F11

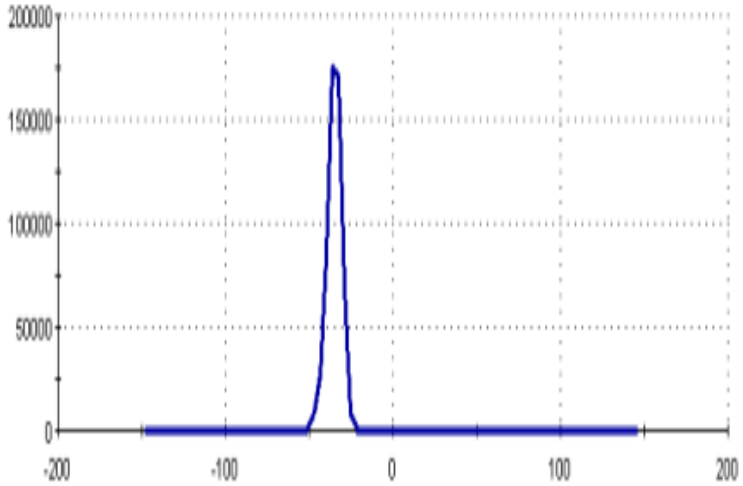


Figure (8) : Zeta potential of formulation F12

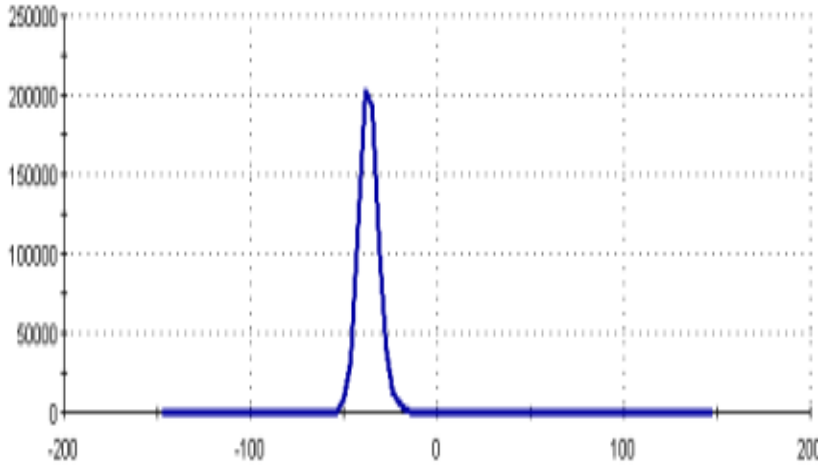


Figure (9) : Zeta potential of formulation F13

Pharmacokinetic Studies

The plasma concentration-time curve for QRT and the prepared nanoparticles is presented in figure 14. it could be observed that $AUC_{0-\infty}$ for the prepared nanoparticles is significantly ($P<0.01$) higher than that of the untreated drug. $AUC_{0-\infty}$ for untreated QRT was found to be 113.411 ng/ml.h, while that of the prepared nanoparticles was found to be 341.219 ng/ml.h which represent 3 fold increases in the $AUC_{0-\infty}$. Since $AUC_{0-\infty}$ is associated with the total amount of drug absorbed, this result confirmed the improvement in the bioavailability of the drug under investigations by preparation of nanoparticles. The value of $AUMC_{0-\infty}$ is, in turn, increased significantly from 374.329 ng/ml.h^2 for the untreated QRT to 1449.63 ng/ml.h^2

This elevation in the area under the curve is associated also with increased the rate of absorption of the prepared nanoparticles with the elimination rate kept constant for both samples. Improving the rate of absorption will shorten the time of onset helping the drug to exert rapidly its therapeutic effect.

The value of Cmax was also increased significantly from 25.28 ng/ml for the untreated

drug to 60.455 ng/ml for the prepared nanoparticles. This improvement is suspected to assist the drug in order to perform its pharmacological action. The value of Tmax is also shortened (insignificantly, P>0.05), while the mean residence time is slightly elevated, as indicated from table 3.

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