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Tripolyphosphate cross-linked curcumin loaded casein nanoparticles to improve water dispersibility, stability and controll delivery

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Abstract
NP's are colloidal structures composed of synthetic or semi synthetic polymers. The aim is to control drug concentration in the target tissues or cells and eliminate undesirable drug level fluctuations in plasma. Curcuminoids, including curcumin-I (diferuloylmethane), curcuminII (monodemethoxycurcumin) and curcumin III (bisdemethoxycurcumin) are natural polyphenolic compounds isolated from the rhizome of turmeric (Curcuma longa). Due to the phenolic groups and conjugated double bonds, curcumin has been observed to possess high cytotoxic activity against tumor cells, strong antioxidant properties and anti - carcinogenesis against a wide range of cell lines. Despite well documented clinical benefits and safety profile, curcumin has limited pharmaceutical role owing to reduced water solubility, instability in alkaline condition, enzymatic degradation, high-metabolism and systemic clearance, hence poor bioavailability (<1%). Several strategies have been explored to enhance curcumin delivery including nanoencapsulation using milk polymers developed as bio-responsive drug delivery systems. Caseins are amphiphilic proteins which self-assemble into stable micellar structures in aqueous solutions. Casein micelles are composed of the four phosphoproteins held together by hydrophobic interactions and by the bridging of calcium phosphate nanoclusters (colloidal calcium phosphate, CCP) that are bound to phosphorylated serine residues of the casein side chains.

Keywords: curcumin, casein, nanoparticles, emulsification, antioxidant, pythochemicals

Introduction

Controlled drug delivery systems exhibit certain degree of control over the drug delivery in the body, and the control may be temporal or spatial or a combination of both [1]. The aim is to control drug concentration in the target tissues or cells and eliminate undesirable drug level fluctuations in plasma. This drug concentration will assure less side-effects and more effective therapies when compared to conventional dosage forms [2]. Drug release from a controlled drug delivery system can be zero-order, variable or bio-responsive. In zero-order release the drug release is constant with time and nearly a constant drug concentration in plasma is maintained over prolong period of time. In variable release the drug is released at variable rates to stimulate natural biorhythms or circadian rhythms. Bio-responsive release is achieved when the drug release is triggered by biological stimulus like changes in pH, concentration of certain biologically active substances like enzymes. In pharmaceutical nanotechnology, several strategies and nanocarriers have been developed to meet these demands.

Pharmaceutical nanoparticles are solid colloidal particles sized between 1 to 1000 nm in diameter used as drug carriers [2]. These carrier materials, with relevant encapsulation strategies that are used to associate bioactive compounds or drugs with nanoparticles. Based on the carrier material, the nanosized controlled release drug delivery systems can be classified 1) Polymeric nanoparticles, 2) Solid lipid nanoparticles, 3) Liposomes, 4) Dendrimers and 5) Polymeric micelles.

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Casein comprises about 94% protein and 6% low molecular weight compounds collectively called colloidal calcium phosphate. Mainly four casein phosphoproteins, αS1-, αS2-, β-, and κ-casein, exist approximately in proportions of 4:1:4:1 by weight respectively in cow milk. Their molecular weights are between 19 and 25 kDa and their average isoelectric point (pI) is between 4.6 and 4.8. All of the four caseins are amphiphilic and have ill-defined structures. Casein may be used in pharmaceutical products either in the form of acid casein which has a low aqueous solubility or sodium caseinate which is freely soluble except near its isoelectric point [3].

Caseins are amphiphilic proteins which self-assemble into stable micellar structures in aqueous solutions.Casein micelles are composed of the four phosphoproteins held together by hydrophobic interactions and by the bridging of calcium phosphate nanoclusters (colloidal calcium phosphate, CCP) that are bound to phosphorylated serine residues of the casein side chains. CCP plays a crucial role in maintaining micellar integrity. Three of the caseins (αS1-, αS2- and β-casein) contain centers of phosphorylation (at least three phosphoserine residues in close proximity) that can bind to the amorphous calcium phosphate cluster. Both αS1- and αS2-casein contain more than one phosphate center and can thus act as linking agents between nanoclusters, Single β-CN molecules have a radius of gyration (Rg) of 4.6 nm and isoelectric pH of 5.33.

Casein proteins have distinct hydrophobic and hydrophilic domains. AS1-casein has a strongly acidic peptide of 40

amino acids that contains seven of the eight phosphate groups, twelve carboxyl groups and only four positive groups. The highly charged N-terminal region of β-casein contains four of the five phosphates of the molecule, seven carboxyl groups and only two positive groups. The sialyated glycoprotein κ-casein has only one phosphate and fourteen carboxylic acid groups located in the hydrophilic C-terminal region called the glycomacropeptide [4].

The interaction between Curcumin (CUR) and casein molecules (CM) was investigated by spectroscopy techniques, which have suggested that CUR forms a complex with CM in the low-polarity regions this complex was efficiently internalized by HELA cells. Interpret of incorporation of CUR into camel β-casein enhanced solubility, cytotoxicity and antioxidant activity earlier evidences have showed that binding ability of casein to CUR significant in pharmacokinetic properties of CUR. The two derivatives of curcumin, Bisdemethoxycurcumin (BDMC) and Diacetylcurcumin (DAC) with β-casein micelle showed the important role of the phenolic OH group in the binding process. It has also been reported that this OH group is important for scavenging oxidants, and connected, with reduced cytotoxicity potential. Caseins have unique properties for fabricating delivery systems of curcumin and pharmaceutical compounds, in addition to being a very important source of calcium and essential aminoacids. As relevant to this study, purified camel β-casein was mixed with curcumin in ethanol, followed by evaporation to remove the solvent and dilution with distilled water. After centrifugation, the supernatant contained 28 μg/mL curcumin. The β-casein can self-associate to form micelles that are capable of dissolving lipophilic compounds but is too costly for food applications. Casein micelles in bovine milk can be dissociated into nanoclusters by high-pressure homogenization, which was used to encapsulate vitamin D2 in nanoparticles of sodium caseinate formed upon depressurization. High-pressure homogenization, however, is currently expensive for food applications. Casein micelles can also be dissociated by calcium ethylenediaminetetraacetate or at alkaline conditions. Curcumin and casein in warm aqueous ethanol may form nanoscale complexes that can be prepared in powdered form by spray-drying for the convenience of transportation storage, and application. Spray drying is a low-cost and scalable technique that has been extensively used for solvent removal and encapsulation [6]

Materials and Methods

Materials

Curcumin (degree of deacetylation ≥ 75%) and span 80 purchased from loba chemicals, Pvt. Ltd. India. Casein purchased from Spectrochem Pvt. Ltd. Mumbai, Sodium Hydroxide and Hydrochloric acid were purchased from Loba chemicals, Pvt. Ltd. Mumbai. Dichloromethane purchased from central drug house (p) Ltd. Delhi

Preparation of Casein Curcumin Nanoparticles

The 3^2 factorial design was empolyed in the intial stages for optimization of formulation. The drug loading was evaluated at 10 levels (1, 2 upto 10ml) of curcumin and span-80 solution in DCM [7] Glycerol at three levels (4, 5,6ml), casein polymer at concentrations (0.2%, 0.4%, 0.6%). Curcumin (2mg\ml) and span-80 (4mg\ml) in dichloromethane was used for drug loading. 0.4% of the casein polymer was selected from 1% of the casein solution prepared (1g of casein in 100ml of 0.1% methyl paraben magnetically stirred followed by the addition of 1.5ml of 1N NaoH stirred for 2hr. Final P^H should be 7. Glycerol was added to the continuously stirred nanoemulsion to prevent coalescence of the nanoglobules produced and rigidisation of interfacial structure by the addition of cross-linking agent (0.5% neutralized tripolyphosphate solution) in different concentrations of 0.2%, 0.3%, 0.5%, 0.6%, 0.75%, 0.9%.

Preparation of stock solutions

Stock solution of Casein

Weighed quantity of (0.1g) methyl paraben to be added to 100 ml 1.5ml of 1N of NaOH in a 250ml beaker, stirred magnetically at 100 rpm for 15min to dissolve. Casein 1g to be sprinkled on the surface of the above solution, stirring continued. Little by little amount of Casein to be added to get lump free polymer solution, pH of Casein solution should be 7.

Solution of curcumin and span 80 in Dichloro methane (DCM): Weighed quantity of 200 mg of Curcumin and 40mg of span 80 to be dissolved in DCM and made up to volume in a 100ml volumetric flask.

0.5% Tripolyphosphate Solution: 0.5g of TPP to be weighed and dissolved in 100 ml of water.

Preparation of curcumin loaded casein nanoparticles

Designated quantities of stock solution of casein 10 ml to be taken in a 50ml beaker. Specified quantities of drug solution 1-10 ml to be added stirred rapidly for 5 min. Specified quantity(6ml) of glycerol to be added and continued stirring. Specified volumes as per formulation design of tripolyphosphate to be added, stirring continued for another 1h and kept aside overnight. The next day nanosuspension to be subjected to ultracentrifugation (Sorval legend XTR, Courtesy – Vignan Bhavan, University of Mysore) at 15000 rpm at 4ºC for 45min. Thus, produced nano plug to be washed twice with double distilled water (DDW).

Preparation of Curcumin unloaded casein nanoparticles

Curcumin unloaded curcumin casein nanoparticles were prepared similar to the procedure outlined in the preparation of loaded nanoparticles without the addition of drug solution. Supernatant and washings collected, acted as blank in the estimation of encapsulation efficiency (% EE).

Preparation of vacuum concentrate of nanoparticulate plugs

Nanoparticulate plug obtained following ultracentrifugation of nanosuspension was separated by decanting the supernatant (1^{st} supernatant) to separate the free drug. This plug was re dispersed in DDW, ultra-centrifuged and the washings mixed with the 1^{st} supernatant. Washing repeated. Supernatant and washings were combined to estimate the free drug, data utilized for calculating% EE. The nano plug thus obtained was subjected to vacuum concentration at 0 ºC, at high pressure (18.1 bars) for 3 h (Savant, Speed vaccum concentrator, SPD 2010). The obtained vacuum concentrate was free flowing powder with good redispersibility.

Table 1: Formulations of Curcumin loaded casein nanoparticles using emulsification by 3^2 factorial design

Formulation codes									
Ingredients	**S1A**	**S1B**	**S1C**	**S2A**	**S2B**	**S2C**	**S3A**	**S3B**	**S3C**
Casein (1%)	**0.1%**			**0.3%**			**0.5%**		
	10ml	**10ml**	**10ml**	**10ml**	**10ml**	**10ml**	**10ml**	**10ml**	**10ml**
Drug B soln(ml)	8	8	8	8	8	8	8	8	8
Glycerol	6	6	6	6	6	6	6	6	6
TPP (0.5%)	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6

Table 2: Data for characterization of curcumin loaded casein nanoparticles

`Evaluation Parameters (± S.D)	**S1A**	**S1B**	**S1C**	**S2A**	**S2B**	**S2C**	**S3A**	**S3B**	**S3C**
Physical stability mean±S.D	30	30	30	30	30	30	30	30	30
% Encapsulation Efficiency mean±S.D	93±5.2	93±4.3	94±3.1	98±4.2	93±2.9	98±4.5	98±3.6	98±4.6	98±2.5
Mean particle Size nm±S.D	1220±3.0	210±0.40	381±6.15	69.60±3.15	110±5.15	1651±7.10	1460±7.1	947±7.10	76±7.10
Zeta Potential mv± S.D	0.1±1.15	0.1±1.1	0.1 ±2.1	143.1 ±2.1	0.1 ±2.15	0.1 ±2.15	0.1 ±2.1	0.1 ±2.1	0.1 ±2.5
%CDR SGF 2H	1.5±0.10	1.7±0.1	1.5±0. 1	1.5±0.100	1.7±0.11	1.5±0. 10	1.6 ±0.5	1.5±0.1	1.7±0.1
%CDR SIF 24H	2.9±0.12	2.6±0.1	28±0.13	2.4±0.101	2.6±0.11	2.6±0.11	2.6±0.1	2.6±0.1	2.6±0.1

(NE) = Not evaluated

Evaluation of Nanoparticals

Melting point determination

Melting point determination of was done by open capillary method. Drug was taken in glass capillary whose one end was sealed by flame. The capillary containing drug was dipped in liquid paraffin inside the melting point apparatus, temperature at which melting was obtained was recorded. Melting point is a good first indication of purity of the sample since the presence of relatively small amount of impurity can be detected by lowering as well as widening in the melting point range.

Fourier Transform Infrared Spectroscopy (FTIR)

Cur –casein Nano plug was subjected for vaccume concentration and there FT-IR transmission spectra were obtained using a FT-IR-8300 spectrophotometer (Shimadzu, Japan). A total of 2% (w/w) of sample, with respect to the potassium bromide disc, was mixed with dry KBR. The mixture was ground into fine powder using an agate mortar before compressing into KBR disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned at 4 mm/s at a resolution of 2 cm over a wavenumber region of 400–4000 cm^{-1} using IR solution software. The characteristic peaks were recorded for different samples Preformulation studies data of drug – polymer interactions are very critical in selecting appropriate polymers. FTIR spectroscopy was employed

To ascertain the interaction of Curcumin with casein[27]. FTIR Spectra of curcumin, casein blank nanoparticles, curcumin loaded nanoparticles and curcumin casein physical mixture were taken. Samples were finely ground with IR grade Potassium bromide and then pressed into pellet, IR spectra were recorded in transmission over the range of 4000-500 cm^{-1} at ambient temperature using Shimadzu FTIR – 8400S.

Determination of λ max (UV Spectroscopy)

10 mg curcumin in 10ml methanol was prepared(1^0 stock solution) 2^0 standard solution of 100micro gm/ml was prepared by suitable dilution containing the concentration 10 microgram/ml was prepared in Methanol and UV spectrum was taken using Shimadzu (UV-2550) double beam spectrophotometer. The solution was scanned in the range of 200-800nm[28]. It shows λ max at 425nm.

Characterization of nanoparticles

Drug loading and encapsulation efficiency

Ten mg of nanoparticulate formulation was triturated in a glass mortar, using aliquots of methanol, quantitatively transferred to 10 ml volumetric flask, made up to volume and shaken overnight on a mechanical shaker at ambient conditions. The mixture was filtered through whatman filter paper. Filtrate diluted to 10 ml suitably with methanol to measure the absorbance at 425 nm using UV spectrophotometer (Shimadzu, Japan, UV- 2450). Theoretical drug loading (%TDL) was estimated using the values of amount of curcumin and other various components present in the formulation.

Percentage drug loading was calculated using the formula,

$$Drug\ Loading(\%) = \frac{Wt.of\ Curmumin}{Wt.of\ formulation} X\ 100$$

Percentage encapsulation efficiency was calculated using formula

$$\text{Encapsulation } Efficacy(\%) = \frac{\%\ Drug\ Loading}{\%\ Theoretical\ Drug\ Loading} X\ 100$$

Swelling (SW %) and release study by pH progression method

Fifty mg of curcumin loaded casein-nanoparticles were taken in pre - moistened and pre weighted dialysis bag(molecular weight, 10 KDa cut off) was immersed in 100 ml of SGF (pH-1.2) containing 0.2% tween 80, stirred magnetically at 50 rpm at 37 ^{0}C for 2 hr. The surface water was blotted with filter paper, weight of dialysis bag with wet nanoparticles was recorded. Degree of swelling was calculated using the equation.

$$SW\ (\%) = \frac{W - W_o}{W_o}\ X\ 100$$

Where W_o and W represent the weight of dry and wet nanoparticles respectively. Absorbance of the receptor medium was measured at λ-max 425nm. Subsequently dialysis bag was shifted to 100 ml of simulated intestinal fluid (SIF) (pH- 7.4) and stirring continued at 50 rpm, at 37 ^{0}C. At the end of 4th h procedure was repeated to record the swollen weights of microparticles and absorbance of receptor medium. Further the swollen weight and absorbance of the receptor medium at the end of 24th h was recorded. Experiments were performed in triplicates.

Particle Size

The mean particle size of the nanoparticle formulations was found to be in the range of 1.1 to 1.7 mm. Increasing concentration of span 80 appears to increase the Nanoparticles size, might be due to bigger micelles produced with increasing concentration. Zeta potential of Nanoparticles found to be increasing with casein concentration.

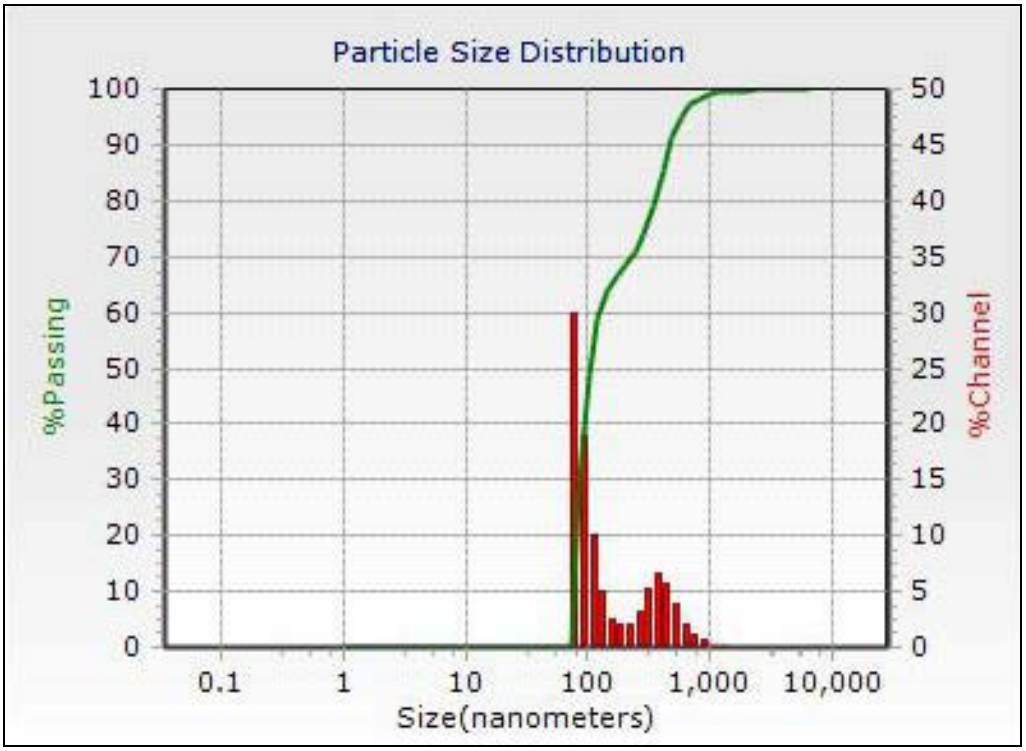
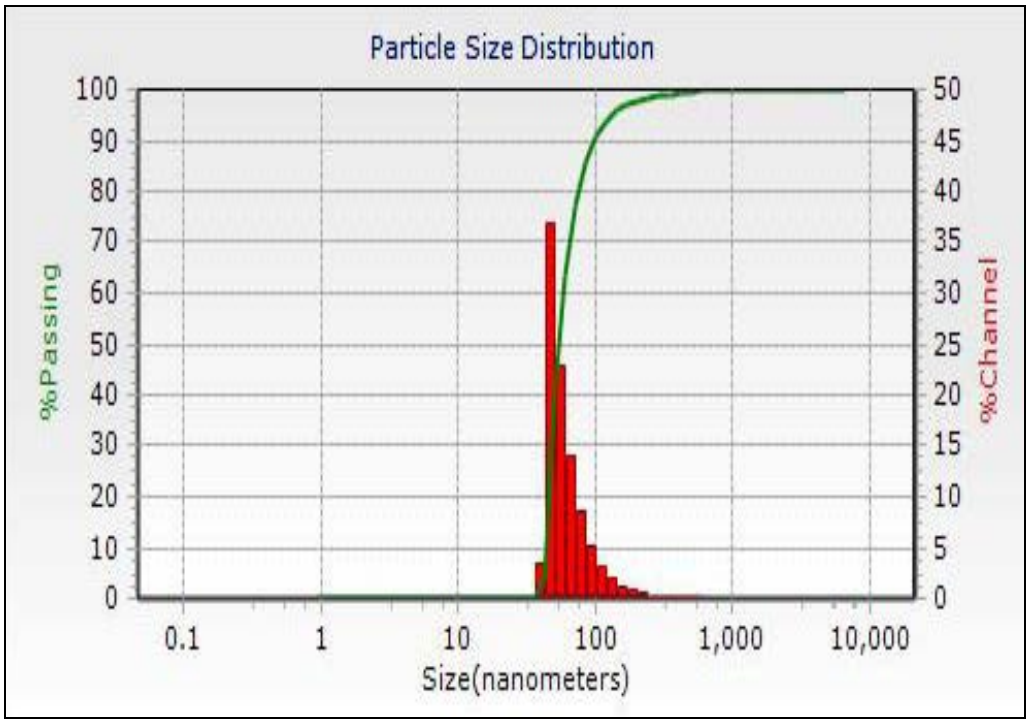
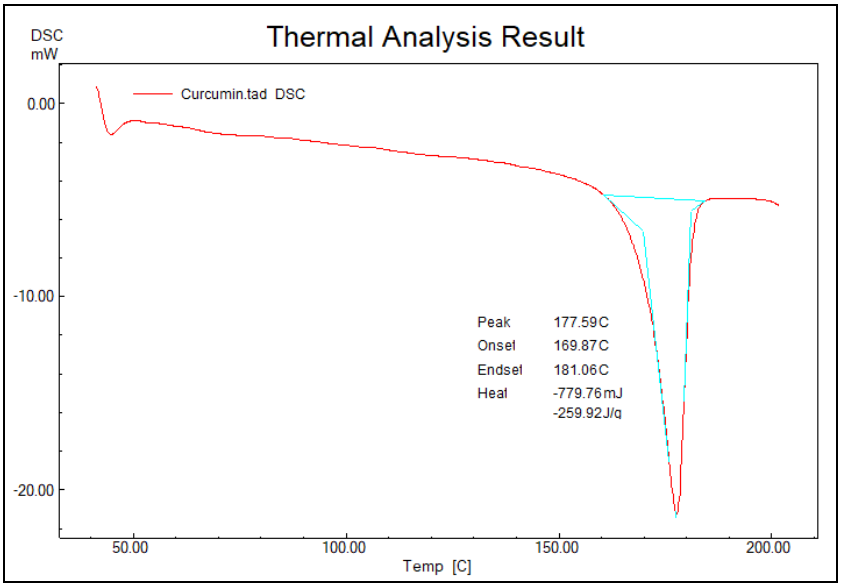


Fig 1: particle size analysis of a) S1a b) S1b



Differential scanning calorimetry



Curcumin

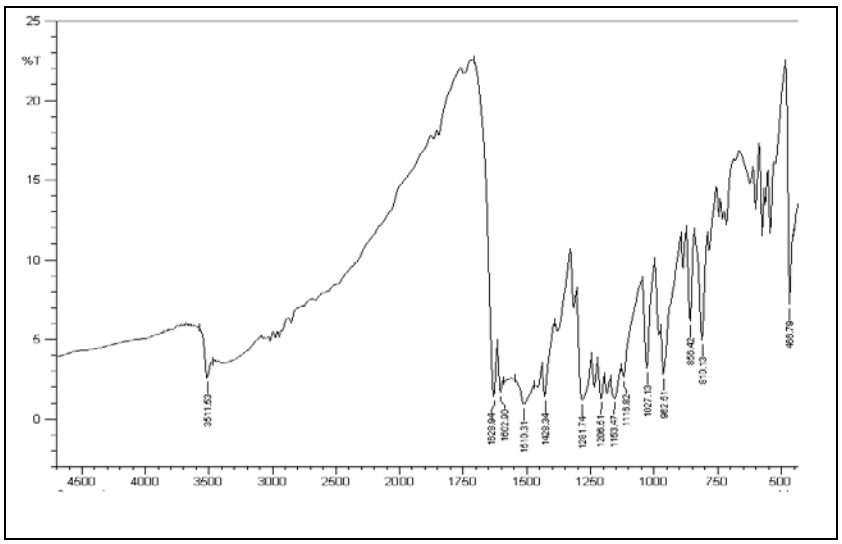


Fig 2: FTIR of sample and curcumin

Results

Pure curcumin spectrum exhibited the characteristic peaks in the intensities of O-H stretch at 3510 cm^{-1}, the C=O stretching frequencies of ester linkage at 1627.

C stretch (aromatic ring) at 1599 cm^{-1}, (CCC) stretch, (CCH) in plane bending, (C-O-C) of aromatic and inter ring chain of pure curcumin at 1427 cm^{-1}, (CCC) stretch and (CCH) in plane bending of the aromatic "keto" part at 1278 cm^{-1}. As these characteristic peaks appeared in the loaded and unloaded nanoparticle formulation with minor shifts in the peaks and confirmed the loading of curcumin into casein in the prepared nanoparticulate system.

	Frequency of pure drug curcumin	**Frequency of nanoparticle formulation 4**
Peak assignments (fig 1)	(cm^{-1})	(cm^{-1})
O-H stretch (C=O) stretching	3510.56	3366.86
frequencies of ester linkage.	1627.97	1636.00
C-C stretch (aromatic ring), CC=0 (in plane bending).	1599.04	1559.24
(CCC) stretch, (CCH) in plane bending, (C-O-C) of aromatic& inter	1427.37	1464.37

To confirm the physical state of curcumin in nanoparticles and to ensure its compatibility with excipients, DSC thermograms were analyzed (Fig. 3a, 3b, 3c, 3d). The thermogram of pure curcumin (Fig. 1) exhibited a sharp endothermic peak at 176.63 ºC, which has previously been attributed to melting of curcumin crystals. The thermogram of casein showed a broad endothermic peak at 82.59 ºC. The peak intensities of the physical mixture was observed at 90 ºC and 157.11 ºC. In the nanoparticulate system there was no evidence of the sharp endothermic peak around 176.63 ºC instead a peak was seen at 133.10 ºC which suggested that the curcumin was in amorphous form, rather than in a

crystalline form and the curcumin molecules have interacted with the casein molecules in the nanoparticulate system.

Stability studies

Temperature stability

Shelf life as a function of time and storage temperature was evaluated by visual inspection of the nanoemulsion system at different time period. aceclofenac nanoemulsion was diluted with purified distilled water to determine the temperature stability of samples. Samples were kept at three different temperature ranges (4°C, room temperature) and observed for any evidences of phase separation, flocculation or precipitation.

Conclusions

The results obtained in this work indicate that developed Nanoparticles formulation, it is now possible to choose the best method of preparation and the best polymer to achieve an efficient entrapment of the drug and it shown better bioavailabilty. From the results, we found in these studies the following conclusions can be drawn:

The developed UV methods were found to be specific, accurate, reliable, and reproducible for quantitative estimation of the drug in vitro. Optimized curcumin casein.

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