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# Formulation and *In Vitro* Evaluation of Multiple Emulsion for An Oral Delivery of Cysteine Protease

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## Abstract

Multiple emulsion is novel approach of drug delivery system for enhancement of bioavailability and pharmacological activity. It is important to prevent the problem of oral drug delivery system and they are stabilized by using of combination of hydrophilic and lipophilic surfactant. In this project the most critical challenge in oral delivery of ficin is to preserve the formulation during its passage through the stomach without denaturation. The key to success of digestive proteins as pharmaceuticals is to have in place an efficient site-specific pH dependent drug delivery system that allows the protein to gain access to the target site at the right time and for an appropriate duration.

**Keywords**

Multiple emulsion, Proteolytic enzyme, Ficin, Eudragit S100 polymer

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**INTRODUCTION:**

Dietary proteins are the essential for growth, repair, and regulation of homeostasis. However, many people are intolerant of such foods, which includes baked beans soup, soyabean, and meat tenderizer. This intolerance can lead to uncomfortable and embarrassing symptoms, such as flatulence, belching, diarrhoea, constipation, malnutrition, food allergies anaemia, undigested food in stool, chronic intestinal parasites, and abnormal flora. These symptoms usually occur during achlorhydria and pancreatin insufficiency. Therefore, the need for a protein – digesting supplement arises. Now a days, the demand for digestive aids has increased, but the supply of pepsin has decreased. Thus plant – derived proteases like papain and ficin is a food grade, highly active endolytic cysteine protease derived from carica papaya and *ficus recemosa* respectively. Its broad substrate specificity and ability to hydrolyse small peptides as well as large peptide protein make ficin and papain an ideal enzymatic supplement. enzymes that play the central role in protein degradation by hydrolysing peptide bonds were known as “proteases” or “peptide hydrolase”.

**Fig.No.1: Ficus Racemosa plant**

**MATERIAL AND METHODS:**

Materials used for research work were as shown in Table no .1.
Instruments used for research work were as shown in Table no 2.

**Table No: -1: List of materials and chemicals**

| Name | Supplier |
|---|---|
| Ficin powder as a proteolytic agent obtained from Raw latex of ficus raecmosa | |
| Ethyl Acetate | Rankem Pvt. Ltd. |
| Methanol | Rankem Pvt.Ltd. Mumbai |
| Eudragit S 100 | Alkem Pvt. Ltd. Mumbai |
| Trifluroacetic acid | S.D. Fine chemicals Mumbai |
| Potassium Dihydrogen Phosphate | S.D. Fine chemicals Mumbai |
| Sodium hydroxide | S. D. Fine chemicals Mumbai |
| HCL | S.D. fine chemicals Mumbai |
| Polysorbate20 | S. D. Fine chemicals Mumbai |
| Distilled water | Lab |
| Disodium EDTA | S. D. Fine chemicals Mumbai |
| Cysteine hydrochloride | S.D. Fine chemicals Mumbai |
| Citric acid | S.D. Fine chemical Mumbai |
| Polyvinyl alcohol | S.D. Fine chemical Mumbai |
| Lactose | S. D. Fine chemicals Mumbai |
| Ethanol | Rankem Pvt.Ltd. |
| Dichloromethane | S.D. Fine chemicals, Mumbai |
| Isopropanol | S.D. Fine chemicals, Mumbai |

**Table No: -2: List of Equipment**

| INSTRUMENT | Model | Manufacturer |
|---|---|---|
| UV-Visible Spectrophotometer | UV-1800 | Shimadzu |
| Hot –air oven | BTI29 | Bio technique, India |
| Electronic Balance | BL-220H | Shimadzu Co. Japan |
| FT-IR | IR-Affinity | Shimadzu Co-Japan |
| Brookfield viscometer | DV3T02346 | Brookfield, USA |
| Magnetic stirrer | IML | Remi India Ltd. |
| Stability chamber | GMP | LABLINE |
| Probe sonicator | F-250 | Frontile Sonicator |
| Digital Weighing balance | AA-2200 | ANAMADE Instrument |
| Centrifugation | - | Biotra centrifuge |
| pH meter | MK VI | Systronics |
| Freezer | GL-A82SPZL | LG Refrigerator |

**Methods:** Solvent evaporation method.

**Table No: -3: Formula of Multiple Emulsion**

| Material | Functional category | Formulation Batches (%w/v) | | | | | | | | Phases volume |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ME1 | ME2 | ME3 | ME4 | ME5 | ME6 | ME7 | ME8 | |
| | | 0.2 | | 0.4 | | 0.2 | | 0.4 | | |
| Cysteine protease ficin | Proteolytic Agent | 0.0199 | 0.199 | 0.398 | 0.398 | 0.199 | 0.199 | 0.398 | 0.398 | W1 0.4 ml Internal aqueous phase |
| Polysorbate 20 3% w/v | Primary emulsifier | 0.006 | 0.006 | 0.012 | 0.012 | 0.006 | 0.006 | 0.012 | 0.006 | |
| Lactose 0.16% w/v | Cryoprotectant | 0.00032 | 0.00032 | 0.00064 | 0.00064 | 0.00032 | 0.00032 | 0.00064 | 0.00064 | |
| D: E: I 5:6:4 | Solvents | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | O Organic Phase 5ml |
| Eudragit S 100 | Acrylic polymer | 0.1 | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.2 | |
| PVA1% w/v solution | Stabilizer | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | W2 External aqueous phase 25ml |
| Lactose 1.6% w/v | Cryoprotectant | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | |
| Total | | | | | | | | | | 30 ml |

D: E: I – Dichloromethane: Ethanol: Isopropanol

**EXPERIMENTATION**

**Method of preparation:** Multiple emulsion was prepared by two step emulsification process: a) Preparation of primary emulsification b) Secondary emulsification.

**Procedure:** 10 ml of distilled water containing an aqueous ficin at a concentration of 2851 mg/ml was prepared. The internal aqueous phase (W1,0.4ml, 0.2 ml) containing ficin, 3% v/v polysorbate 20 (dispersing agent), and 0.16% w/v lactose as a cryoprotectant was emulsified with 5 ml of organic phase for one minute using an ultrasonic disrupter Temperature was maintained at $4^0$C, using an ice bath during emulsification. The organic phase consisted of 200 mg of polymer Eudragit S 100 in 5 ml of a mixed solvent system of dichloromethane, ethanol, and isopropanol in ratio of 5:6:4. The resulting primary emulsion ($w_1$/organic phase) was added drop by drop to the external aqueous (W2 25 ml) of 1% W/V polyvinyl alcohol and 1.6 % w/v lactose solution. Emulsification was continued using an ultrasonic disrupter for two minutes to form a multiple emulsion ($w_1$ /organic phase/$w_2$) at $4^0$c in an ice bath.[1]

**Determination of absorption maxima (λ max) for Ficin:**

The solution of Ficin was found to exhibit maximum absorption at 223nm (shown in Fig 7.5) in phosphate buffer pH6.8 after scanning on the spectrophotometer which was reported as absorption maximum.

**Calibration Curve of Ficin**

A standard solution of 100µg/ml is prepared by dissolving 10 mg of Ficin in 100 ml pH 6.8 phosphate buffer. From the above stock solution, aliquots of 1-5 ml were withdrawn and were diluted with pH 6.8 phosphate buffer up to 10 ml to get solutions of 10, 20,30,40,50 µg/ml concentrations respectively. The resultant dilutions were analysed by UV at 223 nm and absorbance values were noted for each dilution. Finally, a graph of absorbance V/s concentration was plotted.

**Table No: -4: Data for calibration curve of Ficin in phosphate buffer pH 6.8.**

| Sr. No. | Concentration µg/ml | Absorbance at 223 nm |
|---|---|---|
| 1) | 10 | 0.159 |
| 2) | 20 | 0.257 |
| 3) | 30 | 0.376 |
| 4) | 40 | 0.458 |
| 5) | 50 | 0.458 |

**Table No: -5: FT-IR Interpretation**

| Components | Observed peak | Standard peaks | Functional group |
|---|---|---|---|
| Ficin | 1285.30 | 1350-1000 | C-N |
| | 3454.51 | 3500-3100 | N-H |
| | 1265.30 | 1550-1350 | N=O |
| | 1145.75 | 1300-1000 | C-O |
| | 3390.86 | 3400-2400 | O-H |
| | 2357.01 | 2550 | S-H |
| | 1614.42 | 1725 -1700 | COOH |
| | 3441.01 | 3000-2850 | CH3 |
| | 1232.51 | 1465 | CH2 |
| | 3371.27 | 3500-3100 | NH3 |

**Table No: -6: PH of multiple emulsion formulation**

| Sr. No | Formulation code | pH |
|---|---|---|
| 1 | ME1 | 6.2 |
| 2 | ME2 | 6.7 |
| 3 | ME3 | 6.2 |
| 4 | ME4 | 6.8 |
| 5 | ME5 | 6.7 |
| 6 | ME6 | 6.8 |
| 7 | ME7 | 6.7 |
| 8 | ME8 | 7.2 |

**Table No: -7: Melting point of formulation**

| Drug | Melting point |
|---|---|
| Emulsion | $118^0$C |

**Table No: -8: Optimization of stirring time for primary and secondary emulsion.**

| Batches | Stirring time for primary emulsion | Stirring time for secondary emulsion 5min | % phase separation |
|---|---|---|---|
| ME1 | 35 | 5 | 3.44 |
| ME2 | 30 | 5 | 3.72 |
| ME3 | 25 | 5 | 3.44 |
| ME4 | 25 | 3 | No phase separation |
| ME5 | 25 | 6 | 3.44 |
| ME6 | 25 | 3 | No phase separation |
| ME7 | 25 | 2 | 4 |
| ME8 | 25 | 3 | No phase separation |

**Table No: -9: Optimization of stirring speed for primary and secondary emulsion.**

| Batches | Stirring speed for primary emulsion 30 min | Stirring speed for secondary emulsion 5min | % phase separation |
|---|---|---|---|
| ME1 | 1500 | 600 | 4 |
| ME2 | 2000 | 600 | 3.46 |
| ME3 | 2500 | 600 | 3.87 |
| ME4 | 3000 | 600 | 3.46 |
| ME5 | 3500 | 600 | 3.74 |
| ME6 | 4000 | 600 | 0 |
| ME7 | 4500 | 600 | 371 |
| ME8 | 4000 | 600 | 0 |

**Table No: -10: % Entrapment efficiency of Multiple emulsion**

| Sr. No. | Formulation | % Entrapment Efficiency |
|---|---|---|
| 1 | ME2 | 87.54 |
| 2 | ME4 | 83.85 |
| 3 | ME 6 | 88.15 |
| 4 | ME8 | 87.10 |

**Table No: -11: Zeta potential**

| Formulation | Zeta potential (mV) |
|---|---|
| Multiple emulsion ME8 | -**21.71** |

**Table No: -12: % ficin content**

| Formulation Batches | Ficin content |
|---|---|
| ME2 | 0.012 |
| ME4 | 0.015 |
| ME6 | 0.017 |
| ME8 | 0.019 |

**Table No: -13: Partition coefficient**

| Sr. no | Solvent system | Partition coefficient |
|---|---|---|
| 1 | n-tolune/ distilled water | 0.356(Hydrophilic) |

**Table No: -14: *In-vitro* ficin diffusion study**

| Time | Formulations % ficin diffusion | | | | |
|---|---|---|---|---|---|
| (hour) | ME2 | ME4 | ME6 | ME8 | Marketed |
| 1 | 3.60 | 1.38 | 2.55 | 4.82 | 3.12 |
| 2 | 8.37 | 3.31 | 6.706 | 9.70 | 8.20 |
| 3 | 16.74 | 6.54 | 12.01 | 14.99 | 17.19 |
| 4 | 28.48 | 11.58 | 18.54 | 22.44 | 27.41 |
| 5 | 43.68 | 17.64 | 25.86 | 30.57 | 39.45 |
| 6 | 60.63 | 24.41 | 34.79 | 40.60 | 53.81 |
| 7 | 78.20 | 32.37 | 43.86 | 51.55 | 69.86 |
| 8 | 99.28 | 41.09 | 54.86 | 63.64 | 86.30 |

**Table No: -15: % Entrapment efficiency of Multiple emulsion**

| Sr. No. | Formulation Batches | % Entrapment Efficiency |
|---|---|---|
| 1 | ME2 | 91.04 |
| 2 | ME4 | 94.27 |
| 3 | ME 6 | 91.48 |
| 4 | ME8 | 95.38 |

**Table No: -16: Melting point of formulation**

| Formulation Batch | Melting point |
|---|---|
| Multiple Emulsion 6 | 118±10c |

**Table No: -17: Partition coefficient**

| Sr.No | Solvent system | Partition coefficient |
|---|---|---|
| 1 | n-tolune/ distilled water | 0.356 |

**Table No: -18: After Stability Evaluation of Multiple Emulsion formulation**

| Sr. No | Parameter | Temperature conditions | | |
|---|---|---|---|---|
| | | $4^0$-$5^0$C | $25^0$±2C | $40^0$±2C |
| 1 | Physical appearance | No change | No change | No change |
| 2 | pH | 6.8±0.04 | 6.8±0.01 | 6.8±0.03 |
| 3 | Viscosity | 18000±107 | 18000±100 | 18000±100 |
| 4 | Ficin Content | 0.017±0.1 | 0.017±0.1 | 0.017±0.3 |
| 5 | Entrapment efficiency | 91.48±0.2 | 91.48±0.1 | 91.48±0.4 |

(*Mean ± SD n=3)

## RESULT AND DISCUSSION

**Characterization of Ficin by FT-IR:**

Ficin is the proteolytic enzyme which come under the class of endolytic cysteine protease, cysteine containing functional group N-H- having peak range 3500-3100, CH2-1465, CH3-3000-28500, COOO-1725-1700, SH – 2550, NH-33500-3100.

**Viscosity:** Viscosity of multiple emulsion fallows the non-Newtonian flow.

**pH:** pH of all prepared multiple emulsion formulation was determined by using digital pH meter. pH values of multiple w/o/w emulsions containing ficin at to range from 7.2± 0.03 to 6.8±0.04 in samples kept at room temperature. For samples kept at $40^0$c, the pH ranged from 7.2±0.04 to 6.8±0.01 for ME8 and ME6 respectively.

**Melting point analysis**

Melting point of pure drug was found to be $118^0$c with the help Thieles tube containing liquid paraffin and capillary containing formulation.

**% Phase separation:** Formulations (ME1 to ME8) were stored for one week and observed the % phase separation, which was determined by fallowing equation

$$\% \text{ Phase Separation} = \frac{100\ (V_{sep})}{\text{Vol. of batch}} \Big/ \left[\frac{(V1+V2)}{(V1+V2+V0)}\right]$$

V1, V2, V0 represents the volume of internal phase, dispersed phase, and middle oil phase respectively.

**Entrapment efficiency:** Percentage entrapment efficiency is indicative of the percentage of crude drug that has been encapsulated within the polymer powder with respect to the feed drug concentrations. The result of percentage entrapment efficiencies as shown in table no. 7.11 it was found that Ficin could be entrapped with high efficiencies in the formulation due to its lipophilic nature. The average entrapment of Ficin in the formulated multiple emulsion was found to be 87.10 calculated in triplicates.

**Zeta potential determination:**

Zeta potential of the multiple emulsion formulation found to be (shown in fig 7.9) which indicates that formulation is stable.

Zeta potential of the multiple emulsion was analysed by using Malvern zeta sizer. Multiple emulsion was taken in cuvette diluted with distilled water and analysed. Zeta potential observed-21.71 mV, which makes multiple emulsion stable due to the negative charge present.

**Particle size analysis:**

Size distribution analysis was done to determine average particle size of multiple emulsion. The average particle size of optimized multiple emulsion was found to be 55.65 d.nm. From the results of particles size analysis (Figure No.7.10) it can concluded that the particle size of multiple emulsion is homogenous having polydispersity index is 0.391.

**Ficin content determination:**

A 0.5 gm of the prepared multiple emulsion was taken in centrifuge tube containing 5 ml of buffer. Shake the tube for 2 min. 1ml of that stock was withdrawn diluted to 100 ml with buffer and filtered. Total ficin content was determined by UV spectrophotometer at 223nm.

**Partition Coefficient:** Partition Coefficient studies are carried out to find out extent of drug transfer in the aqueous and the other non-aqueous layer. This phenomenon usually is done to obtain the drug concentration in either layer. Partition coefficient value of ficin also revealed its hydrophilic nature which is given in fallowing.

**In- vitro diffusion study:** In vitro release profiles of ficin from its various multiple emulsion formulations are represented in fallowing table7.15. The higher drug release was observed with formulations ME8 and ME6 at pH7 after 8 hours this may be due to presence of maximum conc. of polymer as compared to other formulation.

**Entrapment efficiency:**

Percentage entrapment efficiency is indicative of the percentage of crude ficin that has been encapsulated within the polymer powder with respect to the feed ficin concentrations. The result of percentage entrapment efficiencies as shown in table no. 7.10 it was found that less internal aqueous phase having high efficiencies in the formulation ME6, the average entrapment of Ficin in the formulated multiple emulsion was found to be 91.48% this batch shows good result as compared to other batches.

**Final optimization of Batch:**

Formulation batch was optimized on the basis better results of Ficin content, Entrapment efficiency and In vitro diffusion study which showing better results as compared to other batches so batch ME 6 was optimized.

**Melting point analysis:** Melting point of cysteine protease in batch ME6 was found to be 118±1$^0$C.

**Zeta potential:**

Zeta potential of the batch ME6 multiple emulsion formulation is found to be (shown in Fig 7.11) which indicates that formulation is stable. Zeta potential of the multiple emulsion was analysed by using Malvern zeta sizer. Zeta potential observed -21.71 mV, which makes multiple emulsion stable due to the negative charge present. The general dividing line between stable and unstable suspensions is generally taken at either + 30 or -30 mV. Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable.

**Particle size analysis:**

Size distribution analysis was done to determine average particle size of batch ME6 multiple emulsion. The average particle size of optimized multiple emulsion was found to be 55.65 d. nm and having polydispersity index 0.391 indicate its almost homogeity. The standard range of polydispersity index is 0.15-0.3 indicates reasonable size homogeneity.

**Partition coefficient:** Partition coefficient studies are carried out to find out batch ME 6 extent of cysteine protease ficin transfer in the aqueous and the other non-aqueous layer. Partition coefficient value of ficin also revealed its hydrophilic nature which is given in fallowing table 7.14.

**Stability study:**

Stability study of optimized batch ME6 formulation containing *ficus racemosa* latex powder as proteolytic agent was evaluated, and this study was performed in three different conditions i. e refrigerator, room temperature and oven on 30 days. On these days' time interval were re-evaluation test physical appearance i e colour, odour, and liquefication, pH, and viscosity, ficin content and entrapment efficiency test was given. The results are showing in following table no 18.

Batch ME6 was selected based on result which showing good viscosity, optimum pH, Ficin content, and after stability study there was no change in above mentioned parameter According to this result optimized batch was successfully evaluated the stability study.

**SUMMARY:**

From the first day of collection and purification of raw material i.e. natural latex, the physical appearance suggested that the latex has milky fluid containing proteolytic enzyme which are used in digestion of protein or breakdown of protein. Ficus racemose grows all over India in many forests and hilly areas. It is frequently available around water streams and is also cultivated.

Proteolytic enzymes have great medical and pharmaceutical importance due to their key role in biological process and in the life- cycle of many pathogens. Proteases are extensively applied enzymes in several sectors of industry and Biotechnology, peptide synthesis, digestion of unwanted proteins during nucleic acid purification, cell culturing and tissue dissociation, diagnostic and therapy, Proteolytic digestion of proteins in proteomics.

As ethyl acetate and methanol are used for purification to form free flowing powder characterized by physicochemical property.

In the second phase of research, the formulation development and evaluation of oral drug delivery of Proteolytic enzyme multiple emulsion formulation latex powder i.e. ficin used as active moiety ME6 batch was optimized as compared to other batches having polymer emulsifier ratio is optimum and showing the drug release maximum, stable at different temperature and homogeneous dispersion, showing better result of viscosity, pH.

**CONCLUSION:**

On comparison formulation Batch ME6 can be considered as the best formulation for the preparation of multiple emulsion using Eudragit S 100 polymer, prepared emulsion having small particle size, good stability and maximum entrapment efficiency. The drug release was also sustained upto 8hrs. So batch ME6 pH is compatible with intestine pH and formulation release in the colon because of maximum absorption occur due to large surface area.

Ficin shows extensive proteolytic activity towards proteins, short chain peptides, amino acids, in the field of food and medicine make an ideal enzyme supplement**.**

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