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Full Length Research Paper

Development and validation of a reverse phase high performance liquid chromatography (HPLC) method for determination of tizanidine in human plasma

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A simple and cost effective high performance liquid chromatography (HPLC) method was developed for determination of tizanidine in human plasma using liquid extraction technique. The assay of tizanidine was performed after extraction of drug from plasma using diethyl ether as extraction solvent. The isocratic elution was performed in Agilent, Zorbax SB-C_{18}, 4.6 × 150 mm column maintained at 30°C with mobile phase containing acetonitrile and ammonium acetate in a ratio of 15:85 v/v, respectively. The linear relationship was found within the concentration range of 0.25 to 8 ng/ml, with a flow rate of 1 ml/min and detector wavelength of 230 nm. The evaluated validation parameters were found within the acceptable range. Use of simple HPLC technique with short retention time makes this method a convenient choice for assay of tizanidine in human plasma.

Key words: Tizanidine, bio-analytical method validation, linearity, accuracy, limit of detection (LOD), limit of detection (LOQ), liquid extraction

INTRODUCTION

Tizanidine hydrochloride is an imadazoline derivative that acts on centrally located alpha 2 receptors for producing monolytic response on skeletal muscles (Wagstaff and Bryson, 1997). It is used for the treatment of multiple sclerosis or spinal cord injury or spasticity associated with diseased condition (Sweetman, 2009). Tizanidine is also used for the relieving of pain with disorders like myofacial pain (Meythaler et al., 2001), refractory pain, neuropathic pain, chronic tension type headache and chronic daily headache (Saper et al., 2002). Tizanidine is widely absorbed in gastro intestinal (GI) tract. Peak plasma concentration is achieved in 1 to 2 h after oral administration. Tizanidine protein binding is 30% and it undergoes extensive first pass metabolism (Shanker et al., 2009). Tizanidine is chemically [5-chloro-4-(2-imidazolin-2-ylamino)-2,1,3-benzothiadiazole]and demonstrate basic and lipophilic properties. The drug is ionised in acidic environment and soluble in water (Qi et al., 2003).

Many researchers reported method for determination of tizanidine in human plasma. Lee et al. (2002) evaluated tizanidine by using gas chromatography-mass spectrometry.

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(Lee et al., 2002). Momo et al. (2010) determined tizanidine in human plasma and urine with use of liquid chroma-tography-tandem mass spectrometry in presence of maxiletine (Momo et al., 2010). Ulu et al. (2012) utilized a spectroflourimetric method based on dervitization for estimation of tizanidine in plasma, urine and dosage forms (Ulu et al., 2012).

In the current study, tizanidine hydrochloride was analyzed using simple high performance liquid chromato-graphy (HPLC) method. The low amount of drug in plasma was extracted by liquid-liquid extraction tech-nique. The method overcomes the issue of determination of low concentration of tizanidine in human plasma. The described method is specific and sensitive with short retention time (4.4 min) that makes it cost effective (Figure 1 and Table 1).

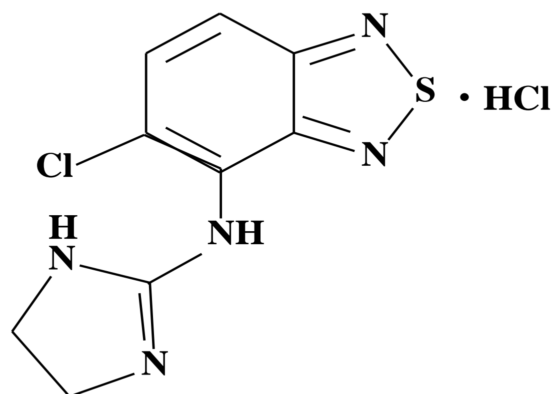

Figure 1. Tizanidine hydrochloride.

METHODOLOGY

Instruments and apparatus

HPLC system (Pump: LC-10 AT VP; detector: SPD-10A *VP*, Shimadzu Corp., Kyoto, Japan), Communication bus module (CBM-102), Guard column C18, 4.0 × 2 mm, HPLC Column (Agilent, Zorbax SB-C18, 4.6 × 150, 5 µm, USA), Column Oven (CTO-10A, Shimadzu Corp., Kyoto, Japan), HPLC Software Class GC 10 version 2.0.0.0, (Shimadzu Corp., Kyoto, Japan), Ultra Sonic bath (Clifton, Nickel Electro Ltd. Somerset, England), Centrifuge machine (Hereues, Osterode, Germany), Vortex mixer (Whirl mixer, England), Filtration assembly (Sartorius, Gorringen, Germany), Swinney Filter assembly (Millipore, England), Hamilton Microliter syringe (Hamilton, Switzerland), Micropippette (Mettler Toledo, Scwerzenbach, England), pH meter (Mettler Toledo, Scwerzenbach, England) were used in the study.

Preparation of chromatographic solutions

Mobile phase

The mobile phase consisted of acetonitrile and 0.1 M ammonium acetate in a ratio of 15:85 v/v. The mobile phase was filtered and sonicated before use.

Preparation of stock solutions and working standard

The standard stock solution was prepared in mobile phase by dissolving accurately weighed quantity of tizanidine to make 1 mg/ml. The standard samples for calibration curve in plasma were prepared by spiking standard stock solution in 1 ml of blank plasma for preparation of secondary stock solution of 100 ng/ml by serial dilution. Secondary stock solution of 100 ng/ml was diluted in plasma for 0.25, 0.5, 1, 2, 4 and 8 ng/ml concentrations.

Sample preparation for drug determination in plasma using HPLC

Sodium fluoride (20 mg) and 0.4 ml of sodium hydroxide (5 mol/L) were added in 1 ml of spiked plasma and vortex for 1 min. Diethyl ether (5 ml) was added and vortex for 10 min for extraction, later centrifuged for 5 min at 4500 rpm. Supernatant was transferred into another test tube and evaporated to dryness using gentle stream of nitrogen at 40°C. The sample was reconstituted in 70 µl of mobile phase, then vortex for 2 min and again centrifuged for 5 min at 4500 rpm. A quantity of 50 µl of the final sample was injected into the HPLC for analysis.

Chromatographic conditions

In the current study, the evaluation of tizanidine was performed at a flow rate of 1 ml/min with detection wavelength of 230 nm and column maintained at a temperature of 30°C.

Method validation

The reported method for determination **of nicergoline metabolite** in human plasma in which Zheng et al. (2012) used tizanidine hydrochloride as internal standard, was modified for estimation of tizanidine hydrochloride in plasma. The proposed method was validated as per International Conference on Harmonisation (ICH) guidelines (ICH, 2005) for selectivity, linearity, accuracy, precision, sensitivity and stability.

Selectivity

Selectivity is helpful for differentiation between drug and other components present in the sample. For selectivity of method, six different blank samples of plasma were run, and it was established at lower limit of quantification (LLOQ) of drug.

Linearity and calibration curves

The linearity was assessed between different concentrations of drug molecule and response of detector by calibration curve (Lister, 2005). The samples with concentrations of 0.25, 0.5, 1, 2, 4, and 8 ng/ml were analyzed in triplicate and calibration curve was constructed, and coefficient of correlation (r^2) was determined.

Intraday and interday accuracy and precision

Five samples of four different concentrations in plasma were analyzed at different time in the same day for intraday accuracy and

Table 1. System suitability parameters.

Parameter	Mean (n=5)	Relative standard deviation (%)	Limit
Retention time (min)	4.4	-	-
Area	22167	0.91	Less than 2
Tailing factor	1.36	0.56	Less than 2
Theoretical plates	6410	-	-

precision determination and analyzed for three consecutive days for interday accuracy and precision. The concentrations were calculated using standard calibration curves.

Lower limit of quantification (LLOQ) and limit of detection (LOD)

Lower limit of quantification (LLOQ) and limit of detection (LOD) were established by analysis of different low concentrations (0.05, 0.1, 0.15 and 0.25 ng/ml). For lower limit of quantification, the signal to noise ratios was observed for the lowest concentration and considered acceptable when response was 7 times of the noise. Lowest detectable and quantifiable concentration against standard was considered as LOQ. The concentration when signal to noise ratio was observed as 3 times has been considered as LOD.

Analytical recovery of method

The absolute recovery was established by comparative study of drug spiked in plasma and in mobile phase. Three different concentrations (2, 4 and 6 ng/ml) each with 5 replicates were examined for determination of recovery.

Plasma stability of the drug

Freeze-thaw and long term stabilities were carried out for the drug in plasma. The freeze and thaw stability was evaluated with selected low (1 ng/ml) and high (7 ng/ml) concentrations with 20 samples of each concentration. Samples were frozen at -20°C for 24 h. A set of all concentrations was thawed and assessed while remaining samples were frozen for the next 24 h. Other two sets of five samples of each concentration were analyzed with the same procedure while the last set was refrozen for next day analysis to complete the three freeze-thaw cycles. These samples were evaluated with reference to freshly prepared samples. The long term stability was performed by preparing fifteen samples of low and high concentrations in plasma. The samples were stored at -20°C. Five samples of each concentration were analyzed at the end of second week and the next five at the end of third week of storage with respect to its initial concentration.

RESULTS AND DISCUSSION

In the current method, tizanidine was extracted from plasma using liquid extraction which is extensively for determination of low drug concentrations in biological fluids (Ciccolini et al., 2001; Esrafili et al., 2007; Xiong et al., 2009). Gan et al. (2002) developed and validated the estimation of tramadol in human plasma using HPLC followed by liquid-liquid extraction. Nirogi et al. (2006) quantified tizanidine in human plasma after liquid-liquid extraction with liquid chromatography tandem mass spectrometry in the range of 50 to 5000 pg/ml. Siddiqui et al. (2011) validated simple HPLC method for simultaneous determination of paracetamol, tizanidine and diclofenac in biological fluids and found a linearity for tizanidine in the concentration range of 120 to 10,000 ng/ml.

Selectivity

Six blank plasma samples were run and no peak at the retention time of drug was detected in plasma. The drug sample was run in same condition and no interference was found. No interfering plasma peak was observed at the drug retention time proved a good selectivity of the method. Selectivity of the method was shown in Figure 2.

Linearity and calibration curves

Evaluations of samples with concentration 0.25, 0.5, 1, 2, 4 and 8 ng/ml were performed in triplicate. The standard calibration curve was linear with a mean r^2 of 0.9989 with percent accuracy between 90 to 104.6%. The linearity chromatogram and linearity curve of tizanidine are shown in Figure 3 and 4.

Accuracy and precision

The HPLC method was also validated for intraday and interday accuracy and precision. The intraday accuracy was found in a range of 90 to 96% while interday accuracy with value of 84 to 92% was observed as shown in Table 2. All the values complied with standard acceptable range of ±15 % for bioanalytical method accuracy and precision.

Lower limit of quantification and limit of detection

The five samples of each concentration (0.05, 0.1, 0.15 and 0.25) were analyzed for LLOQ and LOD determination.

Table 2. Accuracy and precision of tizanidine in plasma.

Parameter	Selected concentrations in method validation (ng/ml)			
	0.25	1	6	8
		Intraday		
Mean (n=5)	0.23	0.9	5.75	7.67
% Accuracy	92	90	95.833	95.875
Standard deviation	0.014	0.039	0.047	0.069
% Coefficient of variation	6.087	4.333	0.817	0.900
		Interday		
Mean (n=5)	0.21	0.85	5.5	7.24
% Accuracy	84	85	91.667	90.5
Standard deviation	0.017	0.043	0.052	0.069
% Coefficient of variation	8.095	5.059	0.945	0.953

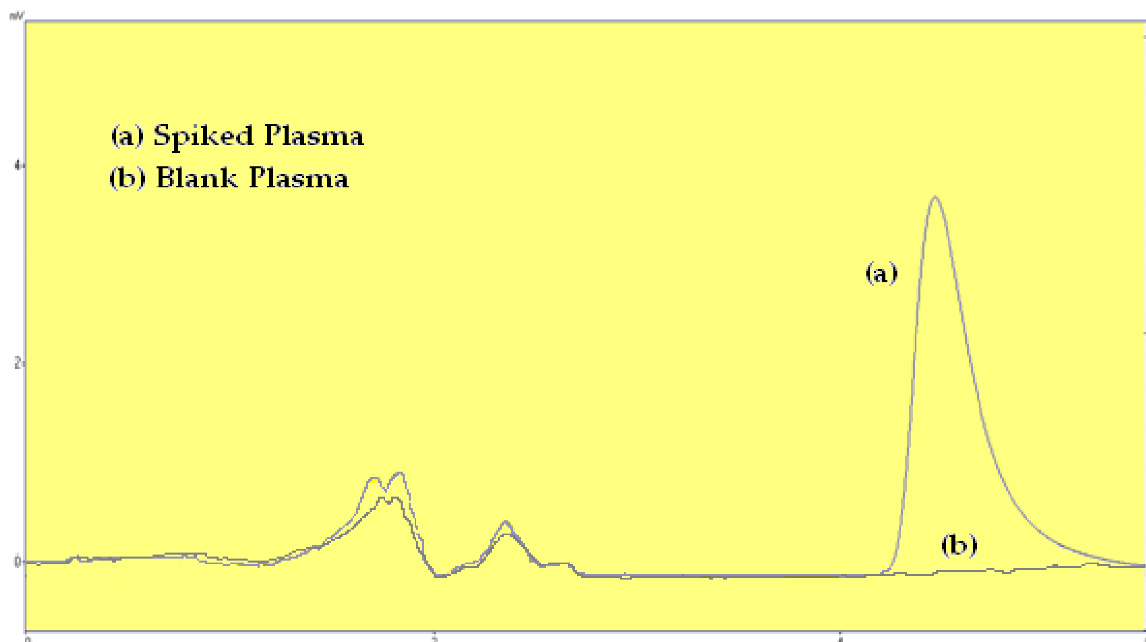

Figure 2. Chromatogram showing selectivity of the method.

The concentration 0.05 ng/ml was not detectable while the lower limit of detection was found as 0.1 ng/ml, and lower limit of quantification (LLOQ) value was 0.25 validated with accuracy of 94.4% that is within the specified limit of 20% and presented in Table 3.

Analytical recovery

Recovery of the method was performed for low, medium and high concentration within the calibration curve range. The method was found with good recovery with the mean analytical recovery of 97.135% for three selected concentrations of 2, 4 and 6 ng/ml as shown in Table 4.

Plasma stability of drug

Freeze and Thaw stability of the drug in plasma were evaluated for three freeze-thaw cycles and estimated accuracy were found to be 99.4, 97.8 and 98.2% for low concentration (1 ng/ml) and 98.66, 98.37 and 98.31% for high selected concentration (7 ng/ml) for freeze thaw cycle 1, 2 and 3, respectively. The average degradation of drug in three FT cycles was found to be 2.268 and 1.040% for concentration of 1 and 7 ng/ml, respectively.

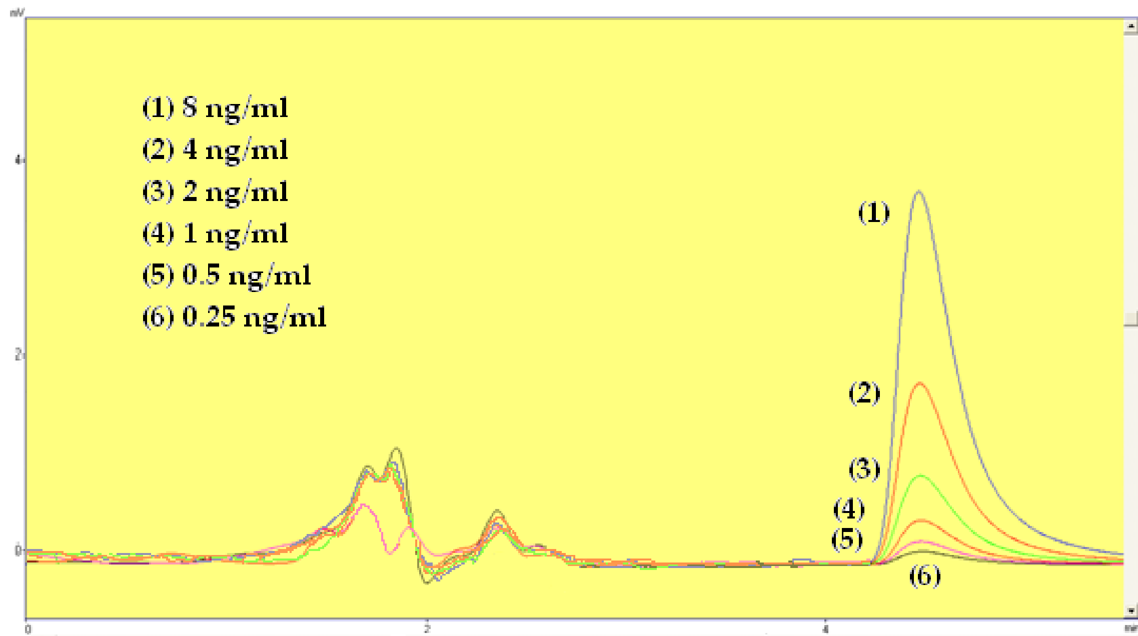

Figure 3. Linearity chromatogram of tizanidine in plasma.

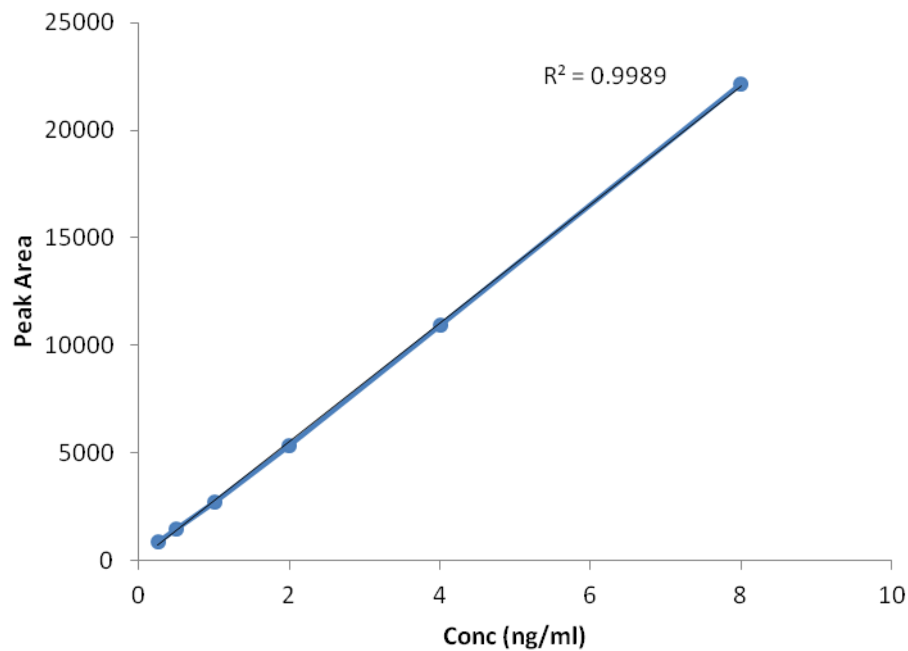

Figure 4. Linearity of different concentration of tizanidine.

Long term stability of the drug was performed for three weeks for low and high concentrations. More than 95% drug was found in the samples after three weeks that represent good long term stability of the drug in plasma.

The mean degradation of drug for both the concentrations of drug in plasma after three weeks was 3.695%. The results of freeze thaw stability and long term stability studies are presented in Tables 5 and 6.

Table 3. Limit of detection of tizanidine in plasma.

Conc. (ng/ml)	Calculated concentration (ng/ml)					Mean	Standard deviation	% Coefficient of variation	% accuracy
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5				
0.25	0.24	0.23	0.23	0.24	0.24	0.236	0.005	2.321	94.4
0.15	0.11	0.1	0.12	0.13	0.11	0.114	0.011	10.002	76
0.1	0.07	0.08	0.06	0.06	0.04	0.062	0.015	23.923	62
0.05	-	-	-	-	-	-	-	-	-

Table 4. Results of recovery studies.

S/No	Conc. (ng/ml)	Mean peak area in plasma (n=5)	Mean peak area in mobile phase (n=5)	% Recovery
1	2	5056.4	5204.6	97.153
2	4	10617.2	11051.6	96.069
3	6	15147.6	15428	98.183
Mean recovery				97.135

Table 5. Freeze and thaw stability of tizanidine.

Parameter	Low concentration (1 ng/ml)				High Concentration (7 ng/ml)			
	Fresh Sample	FT Cycle 1	FT Cycle 2	FT Cycle 3	Fresh sample	FT Cycle 1	FT Cycle 2	FT Cycle 3
Mean (n=5)	1.006	0.994	0.978	0.982	6.98	6.906	6.886	6.882
Standard deviation	0.030	0.021	0.015	0.018	0.027	0.062	0.057	0.066
% Coefficient of variation	2.949	2.086	1.517	1.822	0.392	0.896	0.825	0.955
% Accuracy	100.6	99.4	97.8	98.2	99.71	98.66	98.37	98.31

*FT – Freeze-thaw cycle.

Table 6. Long term stability of tizanidine in plasma.

Parameter	Low concentration (1 ng/ml)			High concentration (7 ng/ml)		
	Fresh sample	After 2 weeks	After 3 weeks	Fresh Sample	After 2 weeks	After 3 weeks
Mean (n=5)	1.012	0.982	1.0	7.032	6.888	6.748
Standard deviation	0.069	0.066	0.069	0.090	0.128	0.113
% Coefficient of variation	6.860	6.693	7.209	1.281	1.858	1.678
% Accuracy	101.200	98.200	95.400	100.457	98.400	96.400

Conclusion

The method has been validated successfully for the determination of tizanidine in human plasma sample. Validation parameters such as selectivity, linearity, accuracy, precision and stability showed good results and complied with standard acceptable range. Hence, this liquid extraction based HPLC method can be used effectively for the estimation of tizanidine in human plasma.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Feasibility study of manufacturing coated tablet lipase from *Yarrowia lipolytica*

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Many studies have provided evidence to use microbial enzyme lipase such as *Yarrowia lipolytica* lipase as substitute of pancreatic lipase. However *Y. lipolytica* lipase lowest resistance at acid pH (1 to 2) limits its application in pharmaceutical industries. In order to improve *Y. lipolytica* lipase resistance to low acid pH, our study was devoted more particularly to coating lipase tablets by Eudragit L30D-55. Lipase tablets were evaluated for weight uniformity, enzyme content, friability, hardness, according to European pharmacopeia. *In vitro* release was studied using pH 1.2 acidic buffer and pH 6.8 phosphate buffer. Study revealed that the prepared tablets were able to sustain enzyme release into the intestine. *Y. lipolytica* lipase tablet was compared to Creon 150 mg (commercial drug). After 3 h incubation, coated tablets did not release lipase at pH 1.2 but was disintegrated totality at pH 6.8 same as Creon 150 mg at 17 min. After 8, 12 and 24 months storage at 25 and 37°C, enzyme tablets retain their enzymatic activity and proprieties.

Key words: Tablet, creon, Eudragit L30D-55, *in vitro* release.

INTRODUCTION

Porcine pancreatic enzymes are the current standard of treatment for pancreatic exocrine insufficiency (PEI), and when protected from exposure to gastric acid, porcine lipase will not be degraded in the stomach. Creon (pancreatin) and pancrelipase are the two primary forms of porcine pancreatic enzyme supplements (PES) available, and pancrelipase has higher enzyme content (Devi et al., 2009).

The Creon is a microgranule enteric capsule content of porcine pancreatic enzymes extract. The Creon microgranules are coated using methylhydroxypropylcellulose phthalate (enteric polymer) (Solvay, pharma). Creon helps to improve digestion of patients with exocrine pancreatic insufficiency due to cystic fibrosis, chronic pancreatitis or after deficient of the pancreas. Bovine enzymes are a potential alternative for individuals who

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refuse to consume porcine products for religious or other cultural reasons (Layer and Keller, 2003). The bovine preparations, however, contain approximately 75% less lipase activity than the porcine and human preparations, and there is some concern about transmittable pathogens (foot and mouth disease and bovine spongiform encephalopathy) from bovine preparations. A very promising approach currently being followed is the replacement of the porcine lipases by microbial lipases.

Microbial preparations of pancreatic enzymes (lipase, protease, and amylase) also exist (Zentler-Munro et al., 1992; Borowitz et al., 2006a). Certain bacteria (for example, *Burkholderia plantarii*) and fungi (for example, *Aspergillus niger*, *Rhizopus arrhizus*) produce pancreatic enzymes with substantial lipolytic activity and greater resistance to gastric acid degradation. The microbial preparations do not require colipase for activation (Layer and Keller, 2003). Of the microbial preparations some fungal lipases are remarkably stable in the acidic gastric milieu, and is rapidly inactivated by bile acids and proteases whereas bacterial lipase has remarkable stability in both gastric and duodenal milieu, particularly in moderately acidic and neutral pH. *In vitro* studies have demonstrated bacterial lipase stability against proteases and bile salts and *in vivo* canine experiments show that bacterial lipase is more potent and efficacious than porcine (PES) (Raimondo and Dimagno, 1994; Suzuki et al., 1997). However, lipase from *A. niger* (Zentler-Munro et al., 1992) and *Yarrowia lipolytica* (Aloulou et al., 2007; Turki et al., 2010a) are some examples of the most tested fungi (pH 3 to 10). The *Aspergillus* and *Yarrowia* lipases have better survival in the duodenal environment as shown *in vitro* and in rats, while the others (*R. arrhizus*) (Layer and Keller, 2003), lipase from *Candida cylindacea* appeared very sensitive to trypsin and to the detergent action of bile salts.

Nowadays in medicine and pharmaceutical domains, acrylate polymers are used to coat drugs. Acrylate polymers and their derivatives, collectively known as Eudragit polymers, were the first synthetic polymers used in pharmaceutical coatings. To form films on the surfaces of pharmaceutical materials, they are usually applied as aqueous polymeric dispersions. Among the additives that are incorporated into the film-forming aqueous polymeric dispersions, the plasticizer is the most critical component. It governs the film formation and the quality of the resulting film. Incorporation of a plasticizer is recommended for polymer coating formulations due to the high glass transition temperatures of polymers. Eudragit L 30D-55, a relatively soft polymer, can be applied in the form of latex under mild working conditions, with the addition of 10 to 20% plasticizer even at room temperature and usually good film formation can be expected (Lehmann, 1997).

Several authors (Turki et al., 2010b) have published works on lipase from *Y. lipolytica* formulated with milk and Arabic gum as good candidate for use in enzyme replacement therapy as a means of treating pancreatic exocrine insufficiency than *Candida rugosa* lipase. Our works proposed preliminary studies to formulate lipase of *Y. lipolytica* on tablet form. This enzyme is stable at pH 3 to 8 (Alloue et al., 2008). However, it is not sufficiently resistant at low pH (1) to be used as a digestive enzyme without protection. This study provides a coating technique of lipase tablets using acrylic polymers such as enteric Eudragit in order to investigate *in vitro* release compared to Creon 150 mg.

MATERIALS AND METHODS

Lipase from *Y. lipolytica* LgX6481 was produced in 2000 L bioreactor (LSL Biolafitte, Poissy, France) in the same conditions as described (Destain et al., 1997). The juice produced was centrifuged on a BTPX205 continuous centrifuge (Alfa Laval, Sweden) at 12,000 × g, at a flow rate of 500 Lh^{-1} and the supernatant was freeze dried for further studies. Eudragit L 30 D-55 (Röhm, Pharma) aqueous colloidal polymer dispersions and diethyl phthalate plasticizer were selected for the study. Silicon emulsion (Vel, Leuven Belgium), talc, lactose DCL11, aerosil 200 and polyplasdone XL were purchased from Merck (Germany). All other chemicals used for the study were of analytical grade. Creon 150 mg (commercial drugs) was purchased in a pharmacy at Gembloux (Belgium).

Tablets manufacture

For 60 g of the total powder was added 30 g of lipase, polyplasdone XL (2.5 g) , 0.2 g of aerosil 200, and finally adding 26.3 g of lactose DCL11. All ingredients was mixed in a mortar and after mixing in a turbulat (System SOHATZ, Switzerland) during 15 min, and 1 g of magnesium stearate was added. The final powder is put in an alternate machine KORCH, to seek the biconvex point (36 tablets/min).

Evaluation of sustained release tablets

In addition, weight variation, hardness, friability, disintegration time, and content uniformity of active ingredient (lipase) were determined for the quality control of Standard test. For the determination of weight variation, 20 tablets were weighed individually on an analytical balance (Sartorious, Germany), and then, mean and standard deviation were calculated. Hardness of 10 tablets were measured using a durometer COMPUTEST (Zurich, Germany). Dusted and accurately weighed 20 tablets were placed in a friabilator (Pharma Test, Germany) and rotated 4 min (100 revolutions). To remove adhering particles, the tablets were dusted again and then weighed. The test for uniformity of mass was performed on 20 tablets (European Pharmacopoeia, 2002). The deviation limit shall be 10% for tablets weighing less than 80 mg. The mean percent friability was calculated from the difference in tablet weights. The disintegration time of tablets (n=6) was determined at 37°C in water using disintegration tester (Komet, Turkey). Lipase was extracted completely from the tablets in pH 6.8 phosphate buffer and the solution was filtered. The enzymatic

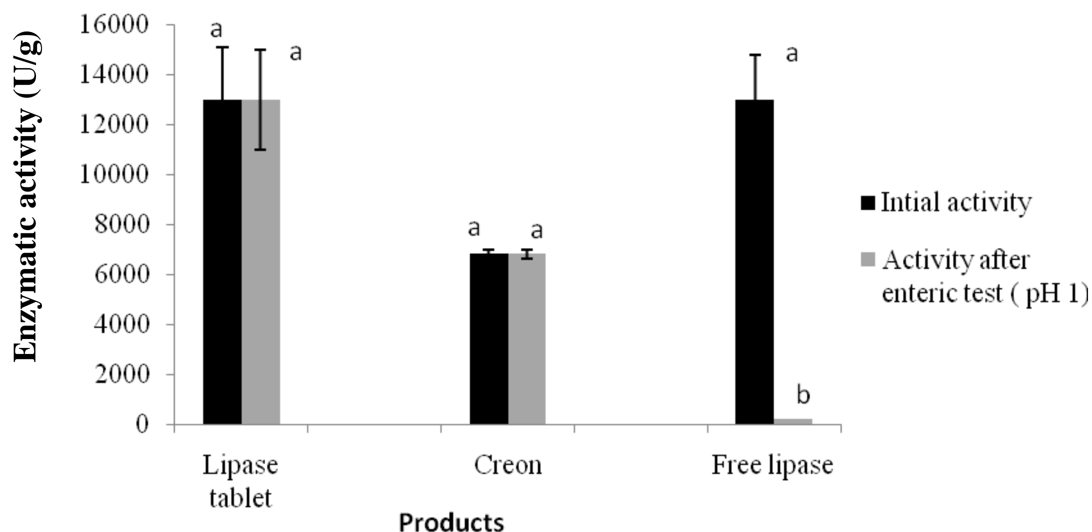

Figure 1. Enzymatic activity of products (free and lipase tablet and Creon) after Gastro-resistant test. For each product, histograms with the same letter are not significantly different, P ≥ 0.05.

activity of lipase was measured by tritimetric method using olive oil emulsion as substrate. After hydrolyse reaction, fatty acid was titrated by NaOH (0.05 N) as described by Destain et al. (1997). Activities are expressed in international units, where 1 U of lipase is the amount of enzyme able to catalyze the release of 1 µmol of fatty acid per minute at pH 7 and at 37°C.

Enteric coating of compressed tablets

In 800 g of water, 1 g of tween 80, 2 g of silicone emulsion and 25 g of talc were dispersed using ultraturax (IKA, Werk) to which was added 333 g of Eudragit L30D-55 and 20 g of diethyl phthalate. After 2 h of stirring, the homogeneous dispersions were obtained. The coating was performed by the fluidized bed drying method (Glatt, Germany) with the inlet temperature and outlet air (45 and 30°C), in an amount of 6 g/min dispersion. After coating, tablets were used for further experiments. Coating was continued till the required mass gain was achieved. *In vitro* dissolution studies were carried out using the basket method. A surplus dissolution test station (Hanson Research Corporation Chatsworth, California, and USA) was used for all dissolution studies. The volume of medium was 500 ml at 37 ± 0.5°C and a stirring rate of 100 rpm was employed. Tablet containing 30 mg of lipase and Creon containing 30 mg of lipase were used for dissolution study. To determine lipase release of the uncoated or coated tablet, a dissolution test (n = 6) was performed using two media with pH 1.2 (HCl, 0.1 M), pH 6.8 (phosphate buffer, 0.5 M), respectively at 2 h for the first and 60 min for disintegration test. For stability studies, the lipase tablets were stored at 25 and 37°C over a period of 12 and 24 months. Samples were evaluated after interval to 1 month, for different parameters such as physical appearance, hardness, mass variation, lipase activity content and dissolution.

Statistical analysis

The data obtained were subjected to analysis of variance(Statistica, 99 Edition) and mean differences determined by Duncan's multiple range test ($P < 0.05$).

RESULTS

Quality control tests

The quantitative evaluation and assessment of a tablet's chemical, physical and bioavailability properties are important in the design of tablets and to monitor product quality. These properties are important since chemical breakdown or interactions between tablet components may alter the physical tablet properties, and greatly affect the bioavailability of the tablet system. The quality control values determined for the test and standard reference are summarized in Table 1.

In vitro release study between Creon and lipase

For comparison study, lipase tablet and Creon capsule were submitted for *in vitro* release test such as enteric test at pH 1 and disintegration test at pH 6.8. We specified that Creon is composed of three pancreatic enzymes such as lipase, amylase and protease but only lipase enzymatic activity is concerned in this work. Lipase activity in Creon 150 mg is 10,000 U which represents 6800 U/g enzymatic activity. Results from Table 2 show that *Y. lipolytica* lipase freeze dried powder can be compressed in the presence of pharmaceutical excipients. After coating, lipase tablets were resistant to gastro-resistant pH (1) at 180 min (3 h). At pH 6.8, tablets were disintegrated at 17 min same to Creon 150 mg and Dry matter is ranged from 95 to 98%. Figure 1 shows clearly that enzyme tablet retains its enzymatic activity after the gastro resistant test at pH (1).

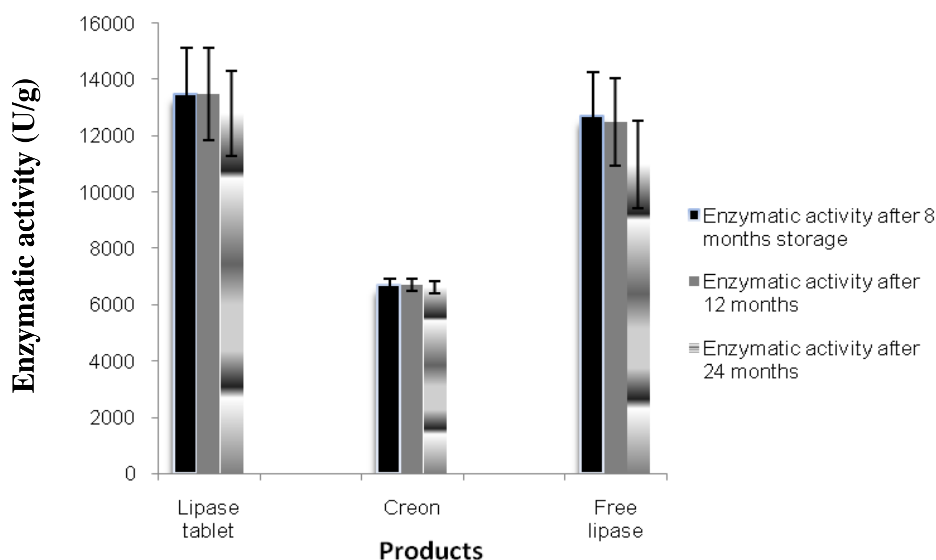

Figure 2. Enzymatic activity of products after 8 to 24 months storage at 25 and 37°C.
For each product, there is no significant difference between enzymatic activities during the storage, P ≥ 0.05.

Table 1. Quality control of lipase tablet.

Quality control	Lipase tablet initial	Lipase tablet after 12 and 24 months at 25 and 37°C	Reference value (Euro Ph, 2002)
Color	White	No change	No change
Hadness (N)	10±3	No change	No change
Uniformity of mass (mg)	60.5±0.08	61.5±0.05	<10%
Friability	0	No change	< 1%
Disintegration time (min)	6±1	No change	<15

Stability studies

The stability study results of *Y. lipolytica* lipase tablets are shown in Table 1. There were no significant changes in their physical appearance, average mass, hardness and lipase activity content. The release profile did not show any significant changes either. Figure 2 shows that after 8, 12 and 24 months storage at 25 and 37°C lipase tablets retain their enzymatic activities.

DISCUSSION

Control quality of tablets study shows that there are various standards that have been set regarding the quality of pharmaceutical tablets. These include weight, hardness, disintegration and dissolution characters. The remaining specifications assure that tablets do not vary from one production lot to another (Liberman et al., 1990). All quality control parameters were within the specified limits (for example, weight variation < 10%, friability < 1% and disintegration time < 15 min).

In vitro release study of product show that *Y. lipolytica* lipase coated tablet can be resistant at pH 1.2 after 3 h and disintegration time after 17 min, similar to creon. This result could be explained by the fact that excipients and film coating are protected tablets against enteric environment and from moisture. Figure 1 shows that *Y. lipolytica* lipase coated tablet and Creon preserve their enzymatic activity at acid pH. It explains that the enteric coating plasticizer of diethyl phthalate and Eudragit $L_{30}D\text{-}_{55}$ were completely insoluble at pH 1.2 and soluble at pH 6.8 while free lipase lost its total activity (significantly different P < 0.05), as previously described by Alloue et al. (2008).

Our results confirm those of Turki et al. (2010b) who have demonstrated that the same free lipase was very sensitive to extreme gastric acidity, and lipase formulated with milk and arabic gum keeps 50% of activity at pH 7 and are resistant to intestinal protease. Tables 1 and 2, and Figure 2 show that all products retain their enzymatic activity at 25 and 37°C. Hence, it can be concluded that

Table 2. *In vitro* release of lipase tablet and Creon capsule before and after storage.

Product	Gastro-resistant test pH 1 (min)	Disintegration test pH 6.8 (min)	Gastro-resistant test pH 1 (min) after 12 and 24 months storage	Disintegration test pH 6.8 (min) after 12 and 24 months	Dry matter after 24 months storage at 25 and 37°C
Lipase tablet from *Y. lipolytica* 30 mg	180±2	17±2	120±1	17±1	98.70±0.07
Creon 150 mg	180±2	16±1	120±1	16±1	99.80±0.01
Free lipase	0	-	0	-	95.35±0.05
Reference values	120	≤60	120	≤60	99.99±0.0

the manufactured tablets were stable and retained their pharmaceutical proper-ties during a period of 24 months. It may suggest that the lipolytic activity is not destroyed in the presence of pharmaceu-tical additives. These results are in accordance with Wilson et al. (2013) which found a good sta-bility after drug storage at room and 40°C temperatures.

Conclusion

Our study shows that tablet lipase from *Y. lipolytica* keep the quasi totality of activity in presence of excipients, and present similar characteristics of pharmaceutical drug (Creon 150 mg). In order to enable the use of this lipase in tablet or other galienic forms, it is very important to perform this study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effect of the alkaline treatment conditions on the tableting performance of chitin obtained from shrimp heads

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Chitin is a natural polymer with a potential pharmaceutical application since its exhibits some biodegradability, biocompatibility and relative non-toxicity. It is obtained from crustacean's exoskeletons by treatment with alkalis and mineral acids followed by a depigmentation step. In this work, the effect of alkaline treatment on tableting properties such as compact tensile strength and disintegration time and other polymer properties, such as acetylation degree and degree of crystallinity was evaluated. A Box Behnken Design with 17 runs, three levels and three factors (that is, reaction temperature at 30, 75 and 100°C, NaOH concentration at 0.5, 3 and 6 M and reaction time at 1, 4 and 8 h) was employed. A combination of a high temperature, alkali concentration and reaction time led to a low chitin yield, reduced its acetylation degree, increased its crystallinity and hence, decreased the tensile strength, but accelerated the disintegration time of compacts. The optimal reaction condition was achieved using an alkali concentration of 2.6 M, temperature of 70°C and a reaction time of 1 h. These conditions rendered compacts with a tensile strength of 2.3 MPa and disintegration of 28.6 min. Therefore, chitin can be used for the preparation of solid dosage forms by direct compression.

Key words: Chitin, deacetylation, alkaline hydrolysis, tableting performance.

INTRODUCTION

Chitin is the second most abundant polymer in nature found in a wide number of invertebrates such as shrimps, lobsters, insects and in the cell wall of yeast and fungi (Campana-Filho et al., 2007). Chitin has an important function comparable to that of cellulose in terrestrial plants. It is structurally identical to cellulose, except for having an acetamide group (-$NHCOOCH_3$) at the C-2 position. It occurs in nature as ordered crystalline microfibrils associated to proteins. It possesses some biodegradability, biocompatibility and non-toxicity (Cho et al., 2000; Synowiecki and Al-Khateeb, 2003). Chitin is extracted by alternating alkaline/acid treatments. The acid treatment is used to dissolve calcium carbonate, whereas the alkaline treatment eliminates proteins. It is also common to employ a final depigmentation step in the purification process (Seodi and Nada, 2007; No, 1995). In chitin, the degree of acetylation (DA) is >50% indicating the presence of some amino groups since some deacetylation might take place during the extraction process (Domard and Rinaudo 1983; Focher et al.,

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Table 1. Experimental matrix for the alkali hydrolysis conditions according to the Box Behnken Design.

Run	Temp (C)	Conc (M)	Time (h)	Yield (%)	AD (%)	DC (%)	TS (MPa)	DT (min)	TP (%)
1	75	3	4	33.9	52.3	66.8	1.32	110	0.6
2	75	3	4	47.1	46.3	62.4	1.68	90	0.08
3	100	3	8	24.5	46.0	60.4	1.68	16.6	0.05
4	100	0.5	4	33.6	52.8	62.1	1.54	28.6	1.4
5	75	0.5	1	40.0	65.3	69.0	2.18	182.8	1.5
6	75	6	1	19.4	56.3	65.3	1.6	10.2	1.6
7	100	3	1	17.6	56.1	60.7	1.53	3.8	1.3
8	75	3	4	36.4	53.4	66.3	1.71	98.7	0.5
9	30	0.5	4	42.8	63.7	36.4	1.64	263	1.8
10	30	3	1	61.0	42.4	46.8	2.53	220	1.3
11	75	3	4	34.65	48.8	65.8	1.48	96.7	0.5
12	30	3	8	34.9	48.2	54.3	2.8	52.5	1.8
13	30	6	4	28.7	55.6	55.7	2.34	38	1.5
14	75	3	4	45	52.8	69.5	1.56	91.8	0.6
15	75	0.5	8	33.8	48.4	67.0	1.61	17.9	0.8
16	100	6	4	23.4	34.7	68.7	1.75	13.7	0.08
17	75	6	8	25.5	16.2	72.5	1.37	0.9	0.2

Temp, Temperature; Conc, concentration; AD, acetylation degree; DC, degree of crystallinity; TS, tensile strength; DT, disintegration time; TP, total protein.

1990). When the degree of acetylation is <50%, this material is regarded as chitosan and becomes soluble in acid solutions (Khor and Lim, 2003; Signini and Campana-Filho, 1999).

Currently, natural or semisynthetic excipients such as microcrystalline cellulose, starch, lactose, calcium diphosphate, mannitol and sorbitol are used to prepare tablets. They are mostly used as diluents for drugs when formulating compacts for immediate release of drugs. These products are prepared by direct compression, dry granulation or wet granulation. However, only microcrystalline cellulose and sorbitol can be used as single excipients for direct compression and dry granulation due to their good mechanical properties. For this reason, it is important to look for new biodegradable and biocompatible excipients as alternative materials to produce tablets. It is possible that the extraction conditions of chitin affect some physicochemical properties varying its tableting performance. Therefore, the objective of this work is to study the effect of alkaline treatment on tableting properties such as tensile strength and disintegration time of compacts and other polymer properties such as degree of acetylation and degree of crystallinity using a Box Behnken Design.

MATERIALS AND METHODS

Shrimp exoskeletons were purchased from Comerpes SA (Cartagena, Columbia). Sodium hydroxide (lot B064398119) was obtained from Merck (Darmstadt, Germany), concentrated hydrochloric acid (lot 3024) and sodium hypochlorite (lot 1791) was purchased from JM chemicals (Medellin, Columbia).

Experimental design

A Box Behnken Design (BBD) with 17 runs was used to assess the effect of alkaline hydrolysis conditions such as temperature (30, 75 and 100°C), concentration (0.5, 3.0 and 6.0 M) and reaction time (1, 4 and 8 h) on yield, acetylation degree, crystallinity and tableting-related properties such as compact tensile strength and disintegration time. A central point in 5 replicate was added to estimate the curvature of the design. The conditions and levels of the alkaline treatment are shown in Table 1. The statistical analysis was conducted using the Design Expert® software 8.04 (Stat-Easy Inc., Minneapolis).

Production of chitin

Approximately, 20 g of dry exoskeletons were milled on a cutting mill (Model 3, Willey, Arthur Thomas Co., Philadelphia, USA), passed through a No. 16 mesh sieve and hydrolyzed at the conditions shown in Table 1 using a heating mantle (P&P, Medellin, Columbia) coupled with a round bottom flask and a two-decked condenser. The solid-to-NaOH solution ratio was (1:10). The dispersion was then neutralized with a 3 M HCl, filtered and dried in an oven (U50, Memmert, Schawabach, Germany) at 100°C for 3 h. The dry material was then treated with a 3 M HCl at 25°C for 24 h to decompose proteins, fats and pigments. This dispersion was then neutralized with a 3 M NaOH, vacuum filtered and treated with a 15% w/v sodium hypochlorite for 24 h at a 1:2 solid-to-NaClO ratio for bleaching. The resulting material was then washed until a conductivity of <20 µS/cm was reached and filtered. The cake thus obtained was dried at 60°C for 24 h and passed through a No. 100

mesh sieve.

Acetylation degree (AD)

Approximately, 1.5 mg of sample was mixed with ~200 mg of dry KBr (previously dried at 110°C for 4 h) with an agate mortar and pestle. The powdered sample was compressed into a pellet using a 13 mm flat-faced punch and die tooling, fitted on a portable press at a dwell time of 5 min. A Perkin-Elmer spectrophotometer (Perkin Elmer, CA, USA) equipped with the Spectra software (Spectrum BX, vs. 5.3.1, Perkin Elmer, Inc, CA, USA) was used to obtain the spectrum between 400 and 4000 cm^{-1}. The resolution, interval length and number of scans employed were 16, 2.0 and 16 cm^{-1}, respectively. The acetylation degree was found by applying the method of Baxter and collaborators by taking the ratio of the absorbance of the FT-IR bands obtained at 1660 and 3450 cm^{-1}, respectively (Baxter et al., 1992).

$$DA = 100 - \frac{\frac{A_{1660}}{A_{3450}}}{1.33} * 100 \qquad (1)$$

where A_{1660} and A_{3450} correspond to the type I amide and hydroxyl stretching band, respectively.

Powder yield

It was determined on a dry basis dividing the amount of chitin that resulted from each run by 20 and multiplying by 100%.

Degree of crystallinity (DC)

It was determined by powder X-ray difractometry conducted over a 5 to 45° 2θ range. A Rigaku difractometer (Miniflex II, Rigaku, Americas, Woodlands, TX) operated at 40 kV and 30 mA equipped with a monochromatic CuKα (α_1=1.5460 Å, α_2= 1.54438 Å) X-ray radiation was employed for the analysis. The sweep speed and step width were 0.5° 2θ/min and 0.008°, respectively. The DC was calculated from the Peakfit software (Seasolve, Inc, Framingham, MA) by separating the crystalline and amorphous scattering radiation using the baseline selection tool.

Protein content (Kjeldahl method)

~0.2 g of sample, a catalyzer (potassium sulfate, copper sulfate and selenium oxide) and 10 ml of concentrated sulfuric acid were added to a round bottom flask and heated up at 100°C for 1 h. This solution was then distilled (~100 ml) in a Buchi automatic distiller and the ammonium borate, thus formed was titrated with 0.01 N HCl. The total protein content was obtained by: Nitrogen×6.25.

Compact tensile strength (TS)

Cylindrical compacts of ~100 mg and 6.5 mm in diameter were made at ~430 psi and a dwell time of 1 s. A single punch tablet press (060804 Compac, Indemec, Itagui, Columbia) equipped with a load cell (LCGD-10K, Omega Engineering, Inc., Stamford, CT) was employed. The data of crushing strength values obtained on a hardness tester (UK 200, Vankel, Manasquan, NJ) were transformed to tensile strength according to the Fell and Newton model (Fell and Newton, 1970).

Compact disintegration time (DT)

Tablets, each weighing ~100 mg, were made on a single punch tablet press (060804 Compac, Indemec, Itagui, Columbia) at ~100 psi using a 6.5 mm round flat-faced punches and die set and a dwell time of 1 s. Three replicate were tested in distilled water at 37°C employing a Hanson disintegrator (39-133-115, Hanson Research Corporation, Northridge, CA, USA) operating at 30 strokes/min.

RESULTS AND DISCUSSION

Surface models

The multiple regression coefficients were higher than 0.9354 indicating that >93.54% of the experimental variance of the properties studied is explained by the surface models and the remaining experimental variation is attributed to random errors (Tables 2 and 3). Therefore, these high r^2 indicate that the cubic models are good predictors for the properties studied and these models described very well the relationship between the three factors (NaOH concentration, temperature and reaction time). These models were also validated by the lack of fit test. Further, the goodness of fit statistic test evaluated whether the variation due to lack of fit of the model was small enough to be accepted as a negligible portion of the pure error. The results showed that the experimental variations observed for the properties studied could be attributed to randomized errors since for all models, the lack of fit was >0.05 and thus the cubic models with three factors can be considered as accurate. The fitted models are:

$1/yield = 0.027+0.013A+0.008B-0.0014C+0.0004AB-0.007AC-0.004BC+0.004B^2+0.007C^2-0.01AB^2$

$AD = 51.2-8.43B-14.25C-3.98AC-5.8BC-3.8C^2+13.2A^2C-7.95AB^2$

$DC = 66.6+5A+0.45B-3.18AB-11.6A^2+1.3B^2+6.0A^2B+4.7AB^2$

$TS = 1.55-0.53A-0.21B-0.2C-0.12AB-0.03AC+0.36A^2-0.089B^2+0.23C^2+0.43A^2B+0.31A^2C+0.36AB^2$

$DT = 99.3-63.9A-53.7B-41.1C+52.5AB+45.1AC+38.9BC-15.7B^2-28.3C^2$

The analysis of variance is shown in Tables 2 and 3. Results indicate that at least one factor was significant for all properties studied. Since only temperature was significant for the degree of crystallinity (DC) and compact tensile strength (TS), it is deducted that these two properties are related and thus, a large arrangement of chitin crystallites had a large contribution on the resulting strength of the tablets. This means that high polymer crystallinity is required for a compact to have a

Table 2. ANOVA analysis of chitin properties.

Source	Sum of squares	Df	Mean square	F- value	p-value, α=0.05
Yield					
Model	0.0018	9	0.0002	11.6	0.002
A-Temp	0.0007	1	0.0007	40.8	0.001
B-Conc	0.0005	1	0.0005	27.1	0.001
C-Time	1.65×10^{-5}	1	1.65×10^{-5}	0.97	0.357
AC	0.0002	1	0.0002	11.8	0.011
C^2	0.0002	1	0.0002	11.9	0.011
AB^2	0.0002	1	0.0002	10.8	0.013
Residual	0.0001	7	1.69×10^{-5}	-	-
Lack of fit	5.9×10^{-5}	3	1.97×10^{-5}	1.3	0.384
Pure error	5.95×10^{-5}	4	1.49×10^{-5}	-	-
Cor total	0.0019	16	-	r^2	0.9372
Degree of crystallinity					
Model	1254.3	7	179.2	18.6	0.000
A-Temp	100	1	100	10.4	0.010
B-Conc	0.8	1	0.8	0.08	0.778
AB	40.3	1	40.3	4.2	0.071
A2	569.3	1	569.3	59.1	<0.000
A2B	72.6	1	72.6	7.5	0.023
AB2	43.7	1	43.7	4.5	0.062
Residual	86.7	9	9.6	-	-
Lack of fit	60.8	5	12.2	1.9	0.28
Pure error	25.9	4	6.5	-	-
Cor total	1341	16	-	r2	0.9354

large TS. Therefore, reaction conditions that produced chitins with a large crystalline component allowed the polymer chains to get closer forming a tight hydrogen bond network, which is boosted upon compaction. As a result, more contact points are formed between particles resulting in tablets of good strength and extended disintegration times. Likewise, soft alkaline conditions let to a product which rendered tablets of a high strength and hence, it was more tortuous for water to penetrate and disrupt the particle-particle interaction and hydrogen bonding in those tablets.

The reaction temperature and NaOH concentration were significant for chitin yield. Basically, NaOH not only hydrolyzed, but denaturalized associated proteins and fats and as a result, a low chitin yield (<25%) was obtained when harsh alkali conditions (that is, 100°C, 3 M and 8 h) were employed. On the contrary, a large chitin yield (~61%) was obtained when mild conditions (that is, 30°C, 3 M and 1 h) were employed.

The models surface plots are depicted in Figure 1. In general, high NaOH concentrations and high reaction temperatures led to a chitin with a low acetylation degree, low yield and high crystallinity which in turn, formed weak compacts and rapid disintegration times.

Degree of acetylation

During the alkaline treatment, degradation of associated proteins and fats along with partial chitin deacetylation took place. Further, a high deacetylation occurred by using harsh reaction conditions such as alkali concentration ≥6 M and temperatures higher than 75°C. Therefore, a high temperature and high NaOH concentration deacetylated chitin independent of the reaction time. Further, the heterogeneous alkaline hydrolysis implied a major deacetylation in the amorphous regions of the polymer leaving partially intact the crystalline native regions of the parent chitin. Therefore, the alkali hydrolysis of the non-crystalline fraction removed the acetamide groups, especially if they are the crystallite surface.

Table 3. ANOVA analysis of chitin tableting properties.

Source	Sum of Squares	Df	Mean square	F Value	p-value,α=0.05
Compact tensile strength					
Model	2.69	11	0.25	9.5	0.011
A-Temp	1.12	1	1.12	43.4	0.001
B-Conc	0.17	1	0.17	6.5	0.051
C-Time	0.16	1	0.16	6.2	0.055
A^2	0.53	1	0.53	20.7	0.006
C^2	0.22	1	0.22	8.5	0.033
A^2B	0.37	1	0.37	14.5	0.013
A^2C	0.19	1	0.19	7.2	0.044
AB^2	0.26	1	0.26	9.9	0.026
Residual	0.13	5	0.03	-	-
Lack of fit	0.03	1	0.03	1.15	0.344
Pure error	0.10	4	0.03	-	-
Cor total	2.82	16	-	r^2	0.9542
Compact disintegration time					
Model	99062.4	8	12382.8	142.5	< 0.0001
A-Temp	32614.6	1	32614.6	375.3	< 0.0001
B-Conc	23058.8	1	23058.8	265.3	< 0.0001
C-Time	13521.9	1	13521.9	155.6	< 0.0001
AB	11035.5	1	11035.5	127	< 0.0001
AC	8127.02	1	8127.02	93.5	< 0.0001
BC	6052.8	1	6052.8	69.7	< 0.0001
B2	1043.0	1	1043.0	12.0	0.009
C2	3385.6	1	3385.6	39	0.0002
Residual	695.2	8	86.9	-	-
Lack of fit	448.2	4	112.0	1.81	0.29
Pure error	247.1	4	61.8	-	-
Cor total	99757.6	16	-	r2	0.9930

Degree of crystallinity

Since upon alkali hydrolysis, chitin swells and NaOH accesses the C-2 acetamide linkage, the net result is an increase of the crystallinity of the samples. This is due to deacetylation taking place in the swollen crystallites. It is possible that the hydrolysis process disrupted the intermolecular hydrogen bond pattern of chitin disturbing the regularity of lateral packing between chains, especially of the most accessible amorphous regions. Once samples are washed and regenerated, the crystalline structure of chitin is increased. During swelling, the intersheet and intra-sheet hydrogen bonds are broken and the crystalline state is disrupted. Once chitin has been solubilized the microfibrillar morphology is lost and the alkali uptake causes swelling without chain scission leading to decrystallization. During swelling, the surface chains are unhinged from the underlying microfibrils and in the subsequent washing treatments, these chains recrystallize on top of the remaining microfibrils.

Chitin yield

In general, chitin yield ranged from 17.6 to 61% on a dry basis. A high yield was obtained at low temperatures and alkali concentrations. This trend was observed independent of the reaction time. In all cases, the total protein content can be considered as negligible. It is possible that during the alkali hydrolysis polymer, degradation is slower in crystalline regions than in amorphous regions. Likewise, at harsh alkaline reaction conditions, deproteinization increased strongly with increasing alkali

Table 4. Validation runs of the optimized alkaline reaction conditions.

Run	Yield (%)	AD (%)	TS (MPa)	DT (min)	DC (%)
Model	21.7	61.8	1.9	30.0	64.1
Experimental	24.6	59.6	2.3	28.6	53.2

AD, Acetylation degree; DC, degree of crystallinity; TS, tensile strength; DT, disintegration time.

concentration rendering a low yield of chitin and virtually no protein left over. This is explained by the increased accessibility of C-2 acetamide linkages to hydroxyl groups due to swelling.

Compact tensile strength and disintegration time

Mild and moderate reaction conditions led to a product with a low acetylation degree, but a more tight hydrogen bond network. This was reflected on a material with better mechanical properties compared to those chitins produced under harsh conditions. On the other hand, large alkali concentrations led to a chitin that rendered tablets of decreased tensile strength. It is plausible that this phenomenon was due to the increased powder crystallinity. For this reason, a high deacetylation eases the formation of inter and intramolecular hydrogen bonds which is needed for the formation of hard compacts. Therefore, more crystalline products are less prompt to form hydrogen bonds with incoming water molecules and thus disintegration times were slower than those of less crystalline chitins.

Validation runs

The optimal reaction conditions were found from the grid search of the models to render a chitin with a degree of acetylation from 50 to 90%, tensile strength >5 MPa and disintegration time <30 min. These responses are expected if a low temperature and concentration and small reaction times are employed. Therefore, compacts with the best tableting properties were obtained when an alkali concentration of 2.6 M, temperature of 70°C and reaction time of 1 h were employed. A validation run was conducted at these conditions and the resulting properties were compared to the theoretical values obtained by the surface models (Table 4). The models with three factors obtained by the BBD experimental design accurately predicted the properties studied.

FT-IR characterization

Chitin chains are organized in sheets where they are tightly held by a number of intra-sheet hydrogen bonds. This tight network is dominated by the strong C-O…..N-H hydrogen bonds where the hydrogen bonds are distributed in two sets with half occupancy in each set. First, a carbonyl group bonds to the hydroxyl group on C-6 and there is also a second hydrogen bond between the OH group on C-3 and the ring oxygen (Guo et al., 2002). The amide groups also forms strong inter molecular hydrogen bond networks (-NH/O]C and –OH/O]C) (Figure 2).

Compared to the original sample, in the chitin spectra, two absorption peaks are observed at 3270 and 3110 cm^{-1} (Figure 3) and were assigned to the N-H stretching by the intermolecular C(2)NH/O]C(7) H-bonds and the O-H stretching by the intermolecular C(6)OH H-bonds, respectively (Liu et al., 2008). Further, a splitting of the amide I vibration at 1660 and 1630 cm^{-1} (minor shoulder) which are assigned to the C=O stretching hydrogen bonded to the N-H groups of the adjacent chain appear bifurcated by forming an additional shoulder attributed to intramolecular hydrogen bonding C=O….$HOCH_2$ (Binias et al., 2007). In contrast, the amide II band is shown at 1560 cm^{-1} and it was attributed to the deformation in the CONH plane (Lamarque et al., 2007). Other minor vibrational bands occurred at 2930 (CH_3 symmetric stretching), 1418 (CH_2 bending and CH deformation), 1375 (C-CH_3 amide stretching), 1314 (amide II and CH_2 wagging), 1155 (C-O-C antisymmetryc bridge stretching), 1073 (C-O-C stretching in ring), 1029 (CO stretching), 896 (C-O-C β-linkage) and 752 cm^{-1}(OH out of plane) (Li et al., 1997).

Compared to the native chitin, the OH bands (3500 to 3200 cm^{-1}) presented great changes. Further, minor changes were observed at the following bands: C-O-C (1000 to 1100 cm^{-1}), C=O (1670 to 1620 cm^{-1}) and the NH amide group (1560 cm^{-1}). The absence of the bands at 1660 and 1630 cm^{-1} in the original native shrimp heads might be due to proteins associated to native chitins. During the alkali hydrolysis, the amide I band at 1660 cm^{-1} gradually increased and was splitted (1630 cm^{-1}). Likewise the band at 1560 cm^{-1} was intensified indicating the prevalence of CONH (amide II). Further, the bands at 3270 and 3110 cm^{-1} were absent in the original sample and were intensified when harsh alkaline conditions were employed.

Figure 1. Surface plots of the properties studied according to the BBD. AD, Acetylation degree; MW, molecular weight; DC, degree of crystallinity; TS, tensile strength; DT, disintegration time.

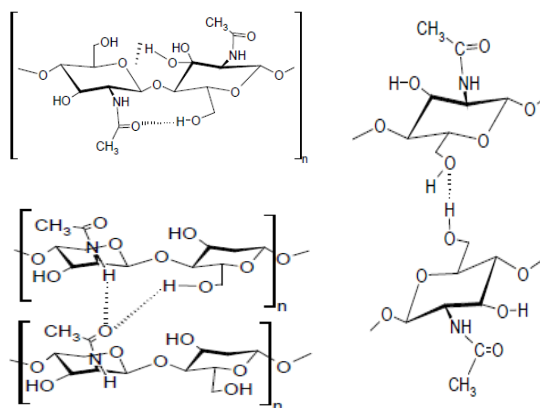

Figure 2. Intra and intermolecular hydrogen network pattern between chitin monomers.

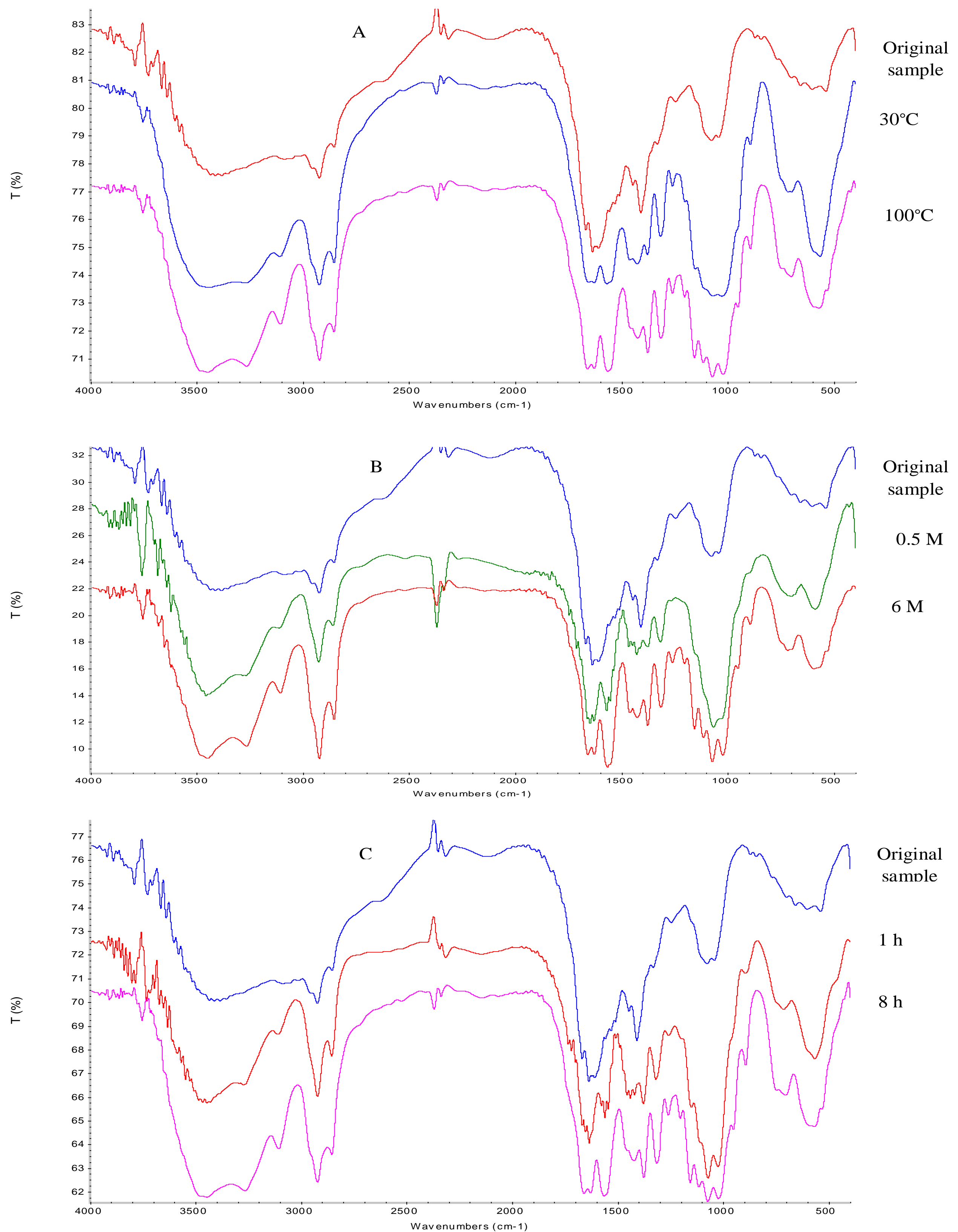

Figure 3. Effect of temperature (A), NaOH concentration (B) and reaction time (C) on the FT-IR chitin bands at 3 M NaOH for 1 h; 30°C for 4 h and 100°C and 3 M, respectively.

Conclusions

The alkaline treatment caused hydrolysis of acetamido groups and deacetylation, forming a different intra and intermolecular hydrogen bonding. At a high alkaline concentration or high temperature native chitin samples obtained from shrimp heads underwent extensive deacetylation. Harsh hydrolysis conditions led to a reduction of compact tensile strength and fastened disintegration time making this material able to be used as excipient for the development of pharmaceutical dosage forms.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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