Ph. D. Thesis

Investigation of proteins during processing into solid dosage forms

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PUBLICATIONS

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2. **Kristó Katalin,** Bajdik János, Márki Árpád, Erős István, Falkay György, Hódi Klára:
   Humán interferon-α -orális szilárd gyógyszerformába történő feldolgozása
   Acta Pharm. Hung. (2008) 78, 31-35.

3. **Katalin Kristó**, János Bajdik, István Erős, Klára Pintye-Hódi:
   Evaluation of the binding effect of human serum albumin on the properties of granules
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4. **Katalin Kristó**, János Bajdik, Peter Kleinebudde, Klára Pintye-Hódi:
   Effect of lubricant on spreading of coating liquid on surface of tablets containing pancreatin
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5. **Katalin Kristó**, János Bajdik, Klára Pintye-Hódi:
   Optimization of the formulation of solid multiparticulate dosage forms containing pancreatin
   Chem. Eng. Res. Des. (2010) 88, 1033-1036.

ABSTRACTS

1. **Kristó Katalin**, Szilárd gyógyszerformákban alkalmazható humán szérum albumin tartalmú köztitermékek előállításának optimálása
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6. **Katalin Kristó**, János Bajdik, István Erős, Klára Pintye-Hódi: Evaluation of the binding effect of human serum albumin on the properties of granules
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7. **Kristó Katalin**, Bajdik János, Peter Kleinebudde, Hódi Klára: Bevonó folyadék szétterülésének vizsgálata különböző összetételű pancreatin tartalmú tabletták felületén
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CONTENTS

1. INTRODUCTION

Biologically active peptides and proteins are increasingly becoming a very important class of therapeutic agents because of their extremely specific activity and high tolerability by the human organism [1]. Their rapid clearance in the body necessitates repeated injections, which is an inconvenient form of therapy, and additionally painful [2]. It is therefore reasonable to formulate dosage forms which can be applied by the patient in a pain-free manner. Alternative routes for the systemic effect are currently becoming widespread. Thus, transdermal, rectal, nasal and buccal therapeutic systems can be used without the destructive effects of the gastrointestinal tract on the proteins [3–5]. The dosage forms can be liquid, semisolid or solid with an appropriate bioadhesive effect [6–8]. The applicability of these solids (e.g. oral tablets for buccal or sublingual use) is easier, and their formulation is therefore a promising method, though with many challenges. The proteins are very sensitive materials, and accordingly their formulation into solid dosage forms is difficult. The pharmaceutical technological methods applied in the case of active agents with low molecular weights for the formulation of solid dosage forms containing proteins are not appropriate as the proteins can be destroyed or their activity can decrease.

Preservation of the activity of proteins and enzymes should be taken into consideration during formulation. This study emphasizes the importance of special aspects in the processing of solid dosage forms containing proteins. Its relevance is constantly increasing because of the spreading of biotechnology and protein-type active agents. In the case of the formulation of solid dosage forms containing proteins the definition of critical control points is important. Process analytical technology (PAT) is currently becoming widely used. The definition of critical control points during the formulation is important in PAT [9-12].

In this study, some examples are demonstrated which must be taken into consideration during the formulation of solid dosage forms containing proteins.

2. AIMS

The two main aims of this study were to investigate of applicability of proteins during pharmaceutical technology prosesses and to investigate the activity of the proteins in the course of processing into conventional solid dosage form. Main purpose was the use of the technologies and apparatuses that can be applied during the formulation of these conventional forms.

The primary objective was the investigation of the applicability of protein as **a** pharmaceutical excipients, in order to evaluate the processibility of a model protein (human serum albumin (HSA)) so as to prepare an intermediate product for an oral solid dosage form. This component can be a carrier of the other protein type active agents [13-15]. If this carrier protein or the active protein enhances the formulation, than its main advantage that the number of other components and/or the possible technological steps can be decreased which can be very useful for a sensitive active agent. Two techniques were applied; a modified layering method and a high shear wet granulation. The adhesive and film-forming effects of HSA solution and the effect of its concentration on the properties of the intermediate product were evaluated. Furthermore, the granulating effect of HSA solution was investigated and compared with those of various conventionally used binding cellulose ethers. The parameters of intermediates which are of importance as concerns tablet making were tested.

The second aim was to evaluate the applicability of proteins as active agents during processing into solid dosage forms. Our purpose was to study the effects of direct compression, wet granulation and coating on the activity of pancreatin (PAN) and pepsin (PEP) as protein-type active components. The activities of these enzymes were investigated during the modelling of circumstances of direct compression and wet granulation. In the first part of this study, the effects of direct compression were evaluated, since heat is generated during compression [16-18]. In our study the thermal stress of the dry material was studied separately, with the objective of an evaluation of the behaviour of this component in the possible “hot spots”. In the second step, the simultaneous effects of moisture and elevated temperature on the activity of the given enzyme were tested by means of a factorial design. Combinations of these factors can occur during granulation and coating. This approach for an assessment of the effects of different factors is not conventional for enzyme-containing dosage forms. This information can broaden the understanding of the effects of different

technological processes, which is indispensable for determination of the critical control point in the preparation of solids containing proteins.

Our purpose was to study the spreading of a coating liquid on the surfaces of different tablets containing PAN and different amounts of microcrystalline cellulose (MCC) and magnesium-stearate (Mg-st.) as excipients, prepared by direct compression. The preformulation compactibility testing is very important. The effects of the components in various compositions were therefore examined with an instrumented tablet machine and the results were compared with those of wettability studies. These results can be applied to evaluate the microstructure of the tablets.

Finally a complex, solid multiparticulate dosage form containing PAN and PEP was also developed for a possible pediatric use. Two intermediate products were prepared and filled into hard gelatine capsule. One of them is minitablets containing PAN with an enterosolvent coating. The other product is granules containing PEP.

3. LITERATURE SURVEY

3.1. Proteins

The application of proteins as active agents is currently increasing because these materials are biodegradable and have specific activity. The proteins and peptides are macromolecules that consist of amino acids and are produced by living organisms. Proteins have a complex internal structure which helps define their biological activity. Any disruption in the primary (amino acid sequence), secondary (two-dimensional structure), tertiary (folding) or quaternary (combination of peptide subunits) structure can result in the deactivation of a protein. Such disruptions may be caused by even the slightest changes in the environment (or even microenvironment) of the protein. The most likely variables which can affect protein structure and stability are related to the temperature, the pH, the solvent, other solutes and the crystallinity states of the protein [19-30]. These problems must be considered during the formulation of dosage forms containing proteins.

3.2. Application of proteins

Biologically active peptides and proteins are increasingly becoming a very important class of therapeutic agents because of their extremely specific activity and high tolerability by the

human organism [1]. Since proteins may be very sensitive, with rapid clearance in the body, the main route of administration is by injection. The injection dosage form, however, has a number of disadvantages, such as low patient compliance, and the possibility of infection and pain during repeated injections [31-41]. This mode of application can be very inconvenient, and the evaluation of non-invasive routes is therefore very relevant.

Accordingly, it is reasonable to formulate dosage forms which can be applied by the patient without pain. Alternative routes for the systemic effect are currently becoming widespread. Thus, transdermal, rectal, nasal and buccal therapeutic systems can be used without the destructive effects of the gastrointestinal tract on the proteins [3–5]. The dosage forms can be liquid, semisolid or solid with an appropriate bioadhesive effect [6–8]. The applicability of these solids (e.g. oral tablets for buccal or sublingual use) is easier, and their formulation is therefore a promising method, though involving many challenges.

Various strategies have been pursued to develop safe and effective oral delivery systems for proteins [42-60] and to devise sustained and long-acting release delivery systems [61-69].

The stability of enzymes is one of the most difficult problems in pharmaceutical technology, in consequence of the many factors involved and the lack of methods permitting an evaluation of their individual contributions.

3.3. Approaches for the formulation of solid dosage forms containing proteins

There are numerous new approaches and techniques for the formulation of proteins into solid dosage forms.

Many of the approaches are based on technologies well known from the formulation of small molecules. In this case the main differences in the formulation of the intermediate and/or final product are the circumstances of production and/or the application of specialized additives. These are very promising approaches for the industry, because they can be applied without developing of new apparatuses. The main purpose of my work was to test the applicability of methods and facilities which are conventionally used in the formulation of solid dosage forms.

3.3.1. Direct compression

The most preferred solid form is the tablet. It has many advantages, its application and administration is convenient, and it can be prepared with high productivity. The consideration

of the application of this form is therefore absolutely reasonable for these active agents as well. Formulation of the tablets can be performed by different methods. The direct compression is one of the preferred ways. For the active agent, it is necessary to consider not only the conventional parameters, such as the properties of the surface, homogeneity, good flowability and good compressibility of the powder mixture [70], but also the effects of high pressure on the internal structure of the active component and the thermal stress generated during high-speed compression performed under high pressure. It is well known that the generation of heat is unavoidable during the preparation of tablets [71, 72]. The heat produced is high for tablets prepared by direct compression, since a high compression force must be applied. It is also well known that if crystals are arranged side to side with a high thermal conductivity edge, then this promotes the attainment of a higher temperature in a very small volume. This increased temperature can be higher than the melting point of the material and the crystals will melt (nearly 100 °C can be reached). Since melted materials recrystallize after compression, the particles lose their individuality. Such sites in the texture are called ”hot spots” [16-18].

The problem with direct compression is the higher compressing force, so that higher temperature can be reached during the compression, which can destroy the proteins. The larger particles of proteins can arrange into ‘‘hot spots’’, where the temperature is higher [16-18]. However, the processibility of small particles is very difficult because of the autoadhesion [73, 74]. The preparation of appropriate intermediates can promote the preparation.

3.3.2. Wet granulation

The preparation of appropriate intermediates can facilitate the preparation of tablets. The most widely used method is wet granulation, in which case separation of the proteins from the liquid is not necessary: the liquid present may serve as a granulating liquid.

Evaluation of the binding effect of the proteins is therefore reasonable before choice of the appropriate composition. If the binding effect of the protein solution is acceptable, the amounts of other excipients can be reduced, which is very favourable because of the sensitivity of proteins.

Moisture and heat can damage enzymes; hence, the application of wet granulation must be considered carefully [75]. Many moisture-sensitive therapeutic compounds are difficult to formulate into commercially deliverable compositions because of their incompatibility with

excipients in wet condition. Not only the granulation process, but also excipients contain high intrinsic moisture and/or have a high propensity for moisture uptake during long-term accelerated stress stability conditions [76-79]. In the case of wet granulation this moisture binding effects of excipients must also be taken into consideration.

3.3.3. Modified layering technique

The intermediate products for tablet making can be different granules, but in special cases particles prepared by the layering technique as well. This layering method is nowadays becoming more popular for the preparation of multiparticulate dosage forms, because conventional granulators (fluidized bed, centrifugal, etc.) can be applied and the small amount of active ingredient can be processed within a short time [80-83]. This technique conventionally uses particles of an inert spherical carrier (e.g. sucrose or cellulose) with the same diameter. The compressibility of these spheres (non-pareils) is not appropriate, and it cannot be changed relevantly with other additives. Accordingly, other carriers must be used for the tablet-making. Powdered cellulose can be a promising component because it is inert and compressible with other cellulose derivatives, but the surface of this component is uneven. This technique is different from the conventionally used layering, but it is also different from granulation, since agglomeration is not an aim.

The choice of appropriate formulation (additives and methods) is therefore very difficult. A method was used which is appropriate for scaling-up. One of the available options is the coating of a carrier in a fluid bed apparatus.

The use of melt granulation is also questionable since proteins and enzymes can then be damaged by the effects of heat.

3.3.4. Multiparticulate dosage forms

Popular multiparticulate systems are minitablets or granules/pellets filled into capsules [84-91]. Different methods are available for the formulation of tablets [92, 93], while a widely-used possibility for the preparation of minitablets is direct compression. The interest in minitablet as dosage forms (filled into hard gelatin capsules or compressed into disintegrating tablets) has recently been increasing continuously, since their multiparticulate nature offers some important pharmacological and technological advantages over conventional single-unit solid dosage forms [94]. Multiparticulate systems allow the possibility of combining several

active components, incompatible drugs or drugs with different release profiles in the same dosage unit [95].

3.3.5. Surface properties

Many pharmaceutical processes involve interactions at interfaces. Interactions between a liquid and a solid are particularly common [74]. Successful film coating requires the spread of a liquid over a tablet surface [93]. The contact angle is used to describe the situation. If a drop of liquid is placed on a flat, smooth, horizontal solid surface, it may spread completely, but it is more likely to form a drop. This drop will exhibit a definite angle relative to the solid, known as the contact angle. The contact angle depends on the compounds involved and the spreading of the components on the surface [96]. Various properties of the liquid, such as its surface tension, polarity, viscosity, etc., can influence this process [97]. The surface of the tablets can also change their wetting [98]. The most important parameters are the surface free energy, surface roughness, porosity, etc.

It is known that several components in the powder mixture can change the surface properties of comprimates [99]. An example of this phenomenon is the enrichment of (Mg st.) on the surface of tablets [100]. The mixing of this component is generally critical. The effects of Mg st. on the microstructure of the tablet surface and hence on the subsequent technological step were evaluated previously, but not all aspects of the coating were fully investigated. This hydrophobization of the surface can cause technological problems during the coating, but it can be useful in the case of a moisture sensitive protein.

The destructive effect of the gastric juice may also be very important [16, 31, 70, 75, 92, 93]. PAN exerts its action in the intestines, and the coating of solid dosage forms with an enteric-resistant polymer is therefore a step very often used in this case [75]. The parameters of this process can influence the efficacy of this enzyme.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Materials for investigation of the applicability of proteins as excipients

4.1.1.1. Model protein

The HSA was used as model protein. A solution containing 39.9 g/l of HSA (and also NaCl, KCl, Na_2HPO_4, KH_2PO_4, etc.) was used in this work (Trigon Biotechnological Ltd., Budapest, Hungary). This liquid was lyophilized at –50 °C and 180-200 mTorr with a freeze-drying machine (Flexi-Dry MP FTS Systems, Inc., Stone Ridge, USA). The lyophilized products were dissolved in the original liquid (39.9 g/l) to produce liquids with contents of 5%, 10%, 12.5%, 15% and 20%.

4.1.1.2. Excipients

In the case of investigation of film-forming effect of HSA ,the carrier was powdered cellulose with an excellent flowability and superior stability (Arbocel A 300, JRS GmbH&Co, Rosenberg, Germany).

Different types of microcrystalline cellulose (MCC) (Vivapur 101, 103 and 105, J. Rettenmaier & Sohne GmbH & Co. KG, Germany) and powdered cellulose (Arbocel P 290, J. Rettenmaier & Sohne GmbH & Co. KG, Germany) were applied in powder mixtures for investigation of the granulation effects of HSA solution. In these investigations mannitol (MAN) (Ph.Eur. Hungaropharma Plc., Hungary) was used as filler.

Aqueous solutions of hydroxypropylmethylcellulose (HPMC, Pharmacoat 606, Shin-Etsu Chemical Co., Ltd., Japan) and hydroxypropylcellulose (HPC, Klucel LF, Hercules Inc., USA) in the same concentration were used in order to compare with the original HSA solution.

4.1.2. Materials for evaluation of the applicability of proteins as active agents

4.1.2.1. Active agents

PAN is a combination of digestive enzymes that is secreted by the pancreas. It is prepared from the pancreas of pig or ox and consists of lipases, amylases and proteases; it is therefore able to break down fats, starch and proteins. PAN (Ph.Eur. Richter Gedeon Plc., Budapest, Hungary) (starch-hydrolysing activity: 18 EPU/mg; proteolytic activity: 3.6 EPU/mg; lipolytic activity: 41 EPU/mg; fat content: 2.2%) was applied as active agent. The moisture content of the untreated PAN was 6.68 % and the average particle size (D50) was 134 μm.

PEP is synthesized in an inactive form by the stomach lining; hydrochloric acid, also produced by the gastric mucosa, is necessary to convert the inactive enzyme and to maintain the optimum acidity (pH=1–3) for the PEP function [93]. The application of acids is therefore necessary. Porcine PEP (Meditop Ltd.) was used in this work. Tartaric acid (Ph. Eur.) and citric acid (Ph. Eur.) were applied as acidifying components.

4.1.2.2. Materials for the enzyme activity measurements

Starch-hydrolysing activity of PAN based on the Ph. Eur. methode. Water-soluble starch (Spektrum-3D, Debrecen, Hungary), pH=6.8 phosphate buffer, 11.7 g/l sodium chloride solution, 1 M hydrochloric acid, 0.05 M iodine solution containing potassium iodide, 0.1 M sodium hydroxide solution and 20% sulphuric acid were applied

Bovine haemoglobin (Sigma-Aldrich), Folin-Ciocalteu reagent (Sigma-Aldrich), trichloroacetic acid (Molar Chemicals Ltd.), hydrochloric acid (Ph. Eur.) and sodium hydroxide (Ph. Eur.) were used for PEP activity measurements.

4.1.2.3. Excipients for the formulations

In this step differential formulation and formulation related tests were performed. Distilled water and 96% ethanol (Spektrum-3D, Debrecen, Hungary) were applied for the study of the effects of wet conditions (can occur during granulation or coating). The excipents for making the tablets containing PAN were MCC (Avicel PH 101, FMC Europe N. V., Brussels, Belgium) and Mg st. (Baerlocher GmbH, Unterschleissheim,(Germany). A 20% aqueous dispersion of Acryl EZE (methacrylic acid-ethyl acrylate copolymer [1:1]) (Colorcon

Ltd., Dartford, Kent, UK) was used as enteric solvent coating liquid. Vivapur 101 was used for PEP granules.

4.2. Methods

4.2.1. Investigation of applicability of proteins as excipients

4.2.1.1. Modified layering technique

The samples were prepared with a fluid bed apparatus (Strea-1, Niro-Aeromatic AG., Bubendorf, Switzerland). The top-spray method was used. The concentration of the HSA solution was varied (see in 4.1.1.1.). The quantity of starting powder and the solid applied in the liquid were the same. Several other factors were also varied for the sample (Table 1). First, powdered cellulose was treated with water to evaluate the effect of water on the aggregation. The constant parameters were as follows.

Quantity of carrier: 100 g
Nozzle diameter: 0.8 mm
Drying temperature: 35 °C
Flow rate: 4 ml/min
Atomizing pressure: 2 bar
Blow-out pressure: 4.5 bar
Preheating: 8 min without atomizing air
Process: in blocks (10-min atomization + 2-min drying)
Last drying: 8 min without atomizing air

Table 1. Parameters in preparation of samples

Sample	Concentration of HSA solution (%)	Quantity of liquid (g)	Total processing time (min)
S0	0	300	134
S1	5	300	130
S2	10	150	65
S3	12.5	120	50
S4	15	100	47
S5	20	75	33

4.2.1.2. High-shear granulation

The influence on different powder mixtures was studied. MAN (as a conventionally used filler such as in buccal preparations) and different types of cellulose were applied. The samples were prepared in a high-shear granulator (ProCepT 4M8 granulator, ProCepT nv, Belgium). The powder mixture was prepared from 100 g of MAN and 100 g of cellulose. The type of cellulose and the composition of the granulating liquid were varied (Table 2). The amount of water was the same for all the samples.

The constant operational parameters were determined in the previous experiments:
Impeller speed: 750 rpm.
Chopper speed: 3000 rpm.
Dosing speed: 5 ml/min.
Spheronization time: 1 min.
Total granulation time: 17 min.
Drying: on trays at 40 °C for 2 h.

Table 2. Compositions of samples

Sample	**Cellulose**	**Liquid**	**Amount of liquid (g)**
G1	MCC 101	4% HSA	80
G2	MCC 101	Water	76.8
G3	MCC 101	4% HPMC	80
G4	MCC 101	4% HPC	80
G5	MCC 103	4% HSA	80
G6	MCC 103	Water	76.8
G7	MCC 105	4% HSA	80
G8	MCC 105	Water	76.8
G9	P290	4% HSA	80
G10	P290	Water	76.8

Particles larger than 2 mm were regarded as waste. The yield calculations and all tests were performed after the removal of these particles.

4.2.1.3. Morphological study

A Hitachi S2400 scanning electron microscope (Hitachi Scientific Instruments Ltd, Tokyo, Japan) was used to determine the shape and the surface of the particles. A Polaron E5100 sputter coating apparatus (Polaron Equipment Ltd, Hertfordshire, England) was applied to induce electric conductivity on the surface of the sample. The air pressure was 1.3-13 mPa.

4.2.1.4. Particle size distribution

The sizes and the size distributions of the samples were evaluated. An analytical sieve (Retsch GmbH, Haan, Germany) was used for testing both types of intermediates. D50 was determined with sieving system software (Retsch EasySieve 2.0). In the case of samples prepared in the fluid bed apparatus, depending on the particle size, the products were divided into 3 groups (<200 μm, 200-315 μm and >315 μm).

4.2.1.5. Flow properties

A powder testing apparatus (PTG-1, Pharma Test GmbH, Hainburg, Germany) was used to test the flow times of 100-ml samples. A teflon accessory 10 mm in diameter. In case of insufficient flowability a stirring at 25 rpm was utilized. Three parallel experiments were performed.

4.2.1.6. Determination of HSA content of layered product

The concentration of HSA was determined with a UV spectrophotometer (Unicam Heλios Alpha, Spectronic Unicam, UK) at 562 nm. The Micro BCA™ Protein Assay (Pierce, Rockford IL, USA) was applied for the determination. The test fluid was phosphate buffer at pH=7.2. The concentrations of active agent in the 3 different groups were determined. Based on the composition the optimum calculated value was 13.04%.

4.2.1.7. Evaluation of granulation liquids

Surface active property of the granulation fluid can influence the efficiency of the granulation. The surface tensions of the HSA, HPMC and HPC solutions and the water were

measured with a ring method (Kruss GmbH, Germany). A Brookfield LVDV-II viscosimeter with a CPE 42 spindle (Brookfield Engineering Laboratories Inc., USA) was used for the determination of the viscosity of the solutions at 25 °C. One-millilitre samples were tested at 12 rpm.

4.2.1.8. Measurement of compressibility

Densities (bulk ($\rho 0$) and tapped ($\rho \infty$)) of both types of intermediates were determined with a STAV 2003 Stampfvolumeter (Engelsmann A.G.L., Germany). Carr's index was calculated from these results [101]; three parallel tests were carried out:

$$\text{Carr`s index} = \frac{\rho_\infty - \rho_0}{\rho_\infty} \times 100 .$$

4.2.1.9. Breaking hardness of granules

The breaking hardness was tested for granules measuring between 710 and 800 μm. The device contains a special specimen holder and a stamp, and is connected to a computer via an interface; thus, not only can the ultimate deformation force be measured, but the process (force–time and force–displacement curves) can also be followed. If the measured plot (force–time) is parallel to the x-axis, the deformation is viscoelastic; if the plot rises linearly, the deformation is elastic. The specimen is located horizontally and the stamp moves vertically (Fig. 1). Twenty parallel measurements were performed.

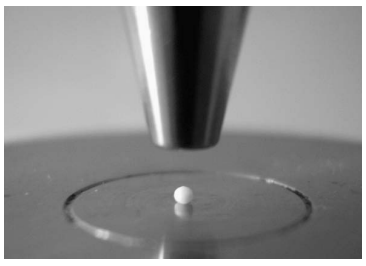

Fig. 1. Sample holder of breaking hardness tester

The measuring range was 0–200 N, the speed of the stamp was 20 mm/min, the output was 0–5 V, and the sensitivity was ± 0.5% ±0.1 digit. The sensor was UNICELL force measuring equipment, calibrated with the C9B 20 kN cell.

4.2.1.10. Water-uptake study

Granulation is not only influenced by the features of the granulation liquid, but also by the wettability of the powder mixture. The Enslin number is a simple semiquantitative measure of the water uptake of a powder, and is equal to the amount of fluid absorbed by 1 g of the powder (ml/g). An Enslin apparatus with a glass filter and a pipette with 0.01-ml accuracy were used for these experiments. A monolayer of particles took up the maximum quantity of water possible through a filter paper under these conditions. Each powder (0.5 g) was tested; 5 parallel experiments were performed. Statistica for Windows 8.1 AGA software (StatSoft, Inc. Tulsa, USA) was used for the statistical analysis. The two-sample T-test was applied for the comparison of two groups of results. The confidence interval was 95% ($p < 0.05$).

4.2.2. Evaluation of the applicability of proteins as active agents

These investigations were performed as preformulation steps. The main aim of this experiment was to evaluate the effect of some technological procedures on the properties of the proteins.

4.2.2.1. Effect of technological process on starch-hydrolysing activity of PAN

The starch-hydrolysing activity of PAN was measured according to Ph. Eur. The concentration of the iodine-starch complex was determined at 576 nm with a UV spectrophotometer (Unicam Heλios Alpha, Spectronic Unicam, UK). The amount of starch hydrolysed was calculated via the amount of the iodine-starch complex. The measurement was performed in consecutive steps. The first mixture contained 1250 µl of a 2% aqueous solution of starch, 500 µl of pH=6.8 phosphate buffer, 50 µl of 11.7 g/l sodium chloride solution and 50 µl of PAN solution. It was incubated for 10 min at 37 °C in a water bath. After this, 100 µl of 1 M hydrochloric acid, 500 µl of 0.05 M iodine solution containing potassium iodide, and 2250 µl of 0.1 M sodium hydroxide solution were added, the mixture was left to stand for 15 min at room temperature, and finally 200 µl of diluted sulphuric acid (20%) was added. The starch-hydrolysing activity was determined; the starch-hydrolysing activity of the untreated PAN was taken as 100%.

4.2.2.2. Measurement of PEP activity

The basis of the analysis of PEP activity was the measurement of the amount of proteins, which cannot precipitate with trichloroacetic acid. The haemoglobin was dissolved in 0.1 M hydrochloride acid solution. The pH was set to 1.6±0.1. The concentration of the haemoglobin solution was 2%. The samples and the untreated PEP powder were dissolved in 0.1 M hydrochloric acid, the pH was set to 1.6±0.1, and the concentration was 0.25%. The incubation time was 10 min at 25 °C. 4% trichloroacetic acid solution was used to precipitate the proteins. The samples were filtered twice through filter paper, leached with 5 ml trichloroacetic acid and dried. After dilution 1 ml of sodium hydroxide and 1 ml of Folin-Ciocalteu reagent were added, and the solution was left to stand for 15 min at room temperature. The relative activity was determined; the activity of the untreated PEP was taken as 100%. The amount of non-precipitating protein was determined at 540 nm with a UV spectrophotometer (Unicam Heλios Alpha, Spectronic Unicam, UK).

4.2.2.3. Determination of effect of direct compression

Both enzyme activity was investigated during the modelling of the circumstances of tabletting. Comprimates 12 mm in diameter were prepared with a hydraulic press (Specac Inc., Graseby) at loads of 2, 4, 6, 8 or 10 t (19.62, 39.24, 58.86, 78.48 or 98.1 kN) from 100% PAN, 100% PEP, 50% PEP-50% tartaric acid and 50% PEP-50% citric acid powder mixtures. The surface of the comprimates was flat. The resulting tablets were pulverized in a mortar before the enzyme activity testing. Not only the effect of the pressure was examined. High pressure is known to induce the generation of heat. This occurs as a very rapid phenomenon during compression. For clarification of this situation, elevated temperatures (40, 50, 60, 70, 80, 90 and 100 °C) were applied in independent tests. In order to study the enzyme activity during elevated temperature, the untreated enzymes was stored for 2 h under dry air conditions in a thermostat (Hereaus Instruments, Hanau, Germany) in which the heat was distributed homogeneously.

4.2.2.4. Determination of effects of wet conditions

The 2^3 full factorial design was applied with 2 central points (Statistica for Windows) to evaluate the effects of the factors on the enzyme activity of PAN and PEP. The factors investigated were temperature, time and liquid content (Tables 3 and 4). The liquid was

ethanol or distilled water for the investigation of the enzyme activity of PAN. During the evaluation of the activity of PEP, the liquid was distilled water and the enzyme activity was studied in three different compositions: 100% PEP, 50% PEP-50% tartaric acid and 50% PEP-50% citric acid. Homogeneous mixtures were prepared in a mortar and the resulting wet masses were stored in hermetically closed containers for a given time. The amount of liquid added to the powder is given as a percentage of the wet mass. The content of liquid utilized during wet granulation is ~10-60%; hence, this range of liquid content was applied in these evaluations. During the calculation of the enzyme activity, the exact liquid contents were considered.

Table 3. Values of factors in measurements of PAN activity

Factor	Low level (-)	Zero level (0)	High level (+)
Temperature (X1)	40 °C	50 °C	60 °C
Time (X2)	1 h	1.5 h	2 h
Content of liquid (X3)	30%	45%	60%

The following approach, involving the interactions of the factors, was used to determine the response surface and the relative effects of the factors (b):

$y=b_0+b_1x_1+b_2x_2+b_3x_3+b_{12}x_1x_2+b_{13}x_1x_3+b_{23}x_2x_3+b_{123}x_1x_2x_3$

Statistica for Windows 8.1 AGA sofware (StatSoft, Inc. Tulsa, USA) was employed for the calculations. During the mathematical evaluations, the confidence interval was 95%, i.e. the differences were significant if $p < 0.05$.

Table 4. Values of factors in measurements of PEP activity

Factor	Low level (-)	Zero level (0)	High level (+)
Temperature (X1)	40 °C	50 °C	60 °C
Time (X2)	1 h	1.5 h	2 h
Content of liquid (X3)	10%	20%	30%

4.2.2.5. Investigation of surface properties of comprimates containing PAN

Distribution of the components during the direct compression is a very important parameter, since an uneven structure can induce additional problems (wettability, compactibility, dissolution etc.). The first group of powder mixtures contained only PAN and MCC in different ratios. The contents of PAN were 0, 10, 20, 30, 40, 50, 60, 80 and 100%.

The second group of powder mixtures contained the same ratios of PAN and MCC, but also 1% Mg st. This concentration of Mg st. is conventionally used in tablet making [93]. In both cases, the PAN and MCC were mixed in a Turbula mixer for 5 min. The lubricant was added to this mixture, and mixing was continued for an additional 1 min. The flat comprimates 12 mm in diameter were prepared with a hydraulic press (Röltgen GmbH & Company KG, Sollingen, Germany) at 1 MPa.

Due to the acid sensitity of PAN, these potential tablets must be coated with intestinosolvent coating. The spreading of 12 µl of Acryl Eze dispersion on the tablet surface was studied via its contact angle. A drop shape analyser was applied (Krüss DSA 10, Krüss GmbH, Hamburg, Germany). The diameter of the needle was 0.8 mm (Sterican 0.8x2.2 mm 21Gx7/8" B. Braun Melsungen AG., Melsungen, Germany). Ten parallel experiments were performed.

4.2.2.6. Calculation of surface free energy

An optical contact angle - measuring device (OCA 20, DataPhysics Instruments GmbH, Filderstadt, Germany) was utilized to determine the wetting properties of the samples. The test fluids were distilled water and diiodomethane (Merck KGaA, Darmstadt, Germany). According to Ström,[92] the dispersion part of the surface tension was 21.8 mN/m for water and 50.8 mN/m for diiodomethane. The polar part of the surface tension was 51 mN/m for water and 0 mN/m for diiodomethane. Compacts of 0.50 g of powder mixtures were made with a hydraulic press (Specac Inc, Graseby, UK), with a dwell time of 10 s, at a pressure of 200 MPa. Circle fitting was applied to determine the contact angle formed on comprimates prepared from different samples.

The microstructure of the tablet surface was predicted from the spreading coefficient, which was calculated from the surface free energy. The indirect method of assessing the surface free energy (γ) from wettability measurements is widely used [102, 103]. In the method of Wu [104], the surface free energy is taken as the sum of dispersive (γ^d) and polar (γ^p) components. The surface free energies of solid materials can be determined by means of contact angle measurements on two liquids with known polarities. They can be assessed by solving an equation with two unknowns:

$$(1+\cos\Theta)\gamma_l = \frac{4(\gamma_s^d\gamma_l^d)}{\gamma_s^d+\gamma_l^d} + \frac{4(\gamma_s^p\gamma_l^p)}{\gamma_s^p+\gamma_l^p}$$

where Θ is the contact angle, γ_s is the solid surface free energy and γ_l is the liquid surface tension.

If the surface free energies of the solid materials are known, the spreading coefficient (S) may be computed and the interactions between the two materials may be predicted. The spreading coefficient is calculated as the difference between the adhesion work and the cohesion work. The two materials which interact can be two powders, a powder and a liquid, or any material and the equipment. The spreading coefficient (S_{12}) of a material (1) over the surface of another material (2) can be determined as follows [105]

$$S_{12} = 4\left[\frac{\gamma_1^d \gamma_2^d}{\gamma_1^d + \gamma_2^d} + \frac{\gamma_1^p \gamma_2^p}{\gamma_1^p + \gamma_2^p} - \frac{\gamma_1}{2}\right]$$

4.2.2.7. Study of compactibility

Based on the preliminary results, the effects of the excipients on the compactibility were also studied. Pure PAN, and powder mixtures containing 50% PAN with or without Mg st. were compressed into tablets with a Korsch EK0 instrumented eccentric tablet machine (Emil Korsch Maschinenfabrik, Germany). The strain gauges allow the pressure forces on the upper and lower punches to be followed with force-measuring equipment. The displacement transducer was fitted over the upper punch. The air temperature was 24-25 °C and the relative humidity was 45-50%.

The lubrication coefficient (R) can be calculated from the upper (F_{upper}) and lower (F_{lower}) forces [106]. This coefficient refers to the loss of force due to the internal and external friction:

$$R=F_{lower}/F_{upper}$$

The plasticity was calculated according to Stamm and Mathis (PL_{S-M}) [107]:

$$PL_{S-M} = \frac{E_2}{E_2 + E_3} \times 100$$

The work of friction was also determined from the detected values. De Blaey and Polderman [108] defined the work of friction as the integral of the difference between the upper and lower punch forces. Later, Järvinen and Juslin [109] presumed that the movement of the particles varies linearly with the distance of the upper punch. According to the Unckel equation [110], the distribution of the axial force decreases exponentially from the upper to the lower punch:

$$W_{fric} = \int_{s1}^{s2} \frac{F_U - (F_U - F_L)}{\ln F_U / F_L} ds$$

where the height of the column changes from s1 and s2 during the compression, F_U is the upper punch force and F_L is the lower punch force [111].

Statistical tests

Statistica for Windows 8.1 AGA software (StatSoft, Inc. Tulsa, USA) was used for the statistical analysis. The two-sample T-test was applied for the comparison of two groups of results. The confidence interval was 95% ($p<0.05$).

4.2.3. Formulation of solid dosage form containing proteins

4.2.3.1. Preparation and investigation of the coated PAN minitablet

In the first step, the preformulation study was carried out. The flowing time, the moisture content, the average particle size and the compressibility were measured, and the Carr index and the Hausner factor were calculated. Based on the previous results the powder mixture contained 50% PAN, 49% Avicel 101 and 1% Mg st., and was homogenized in the Turbula mixture. Tablets 3 mm in diameter were prepared with a Korsch EK0 eccentric tablet machine (Emil Korsch Maschinenfabrik, Berlin, Germany). The temperature was 28 °C and the relative humidity was 48%. The average mass of the tablet was 18 mg. The minitablets were coated with Acryl EZE dispersion with the Wurster method. The coating liquid was contained 20% Acryl EZE, 0.2% simeticon emulsion, 0.2% indigo carmine and distilled water.

Quantity of carrier: 200 g
Nozzle diameter: 0.8 mm
Drying temperature: 40 °C
Flow rate: 4 ml/min
Atomizing pressure: 2 bar
Blow-out pressure: 4.5 bar
Preheating: 3 min without atomizing air

The average mass, diameter and height, the breaking force with the Heberlein apparatus (Flisa, Le Locle, Switzerland), the friability (Erweka GmbH, Heusenstamm, Germany), the disintegration time in distilled water and the enzyme activity of the minitablet without coating

were investigated. After the coating, the average mass, diameter and height, and the disintegration time in artificial gastric fluid (pH=1.2) and in phosphate buffer (pH=6.8) were investigated. A dissolution tester (Erweka DT 700, Heusenstamm, Germany) with a paddle was used for the PAN content measurement and the dissolution study. The coated minitablets containing PAN (320 mg) were placed into the basket of the dissolution tester. In the first step, the coating in artificial gastric fluid (pH=1.2) was checked after the dissolution of PAN was measured in phosphate buffer (pH=6.8). The dissolution medium consisted of 900 ml of artifical gastric fluid (pH=1.2) kept at 37.0 ± 0.5 °C. The rotational speed of the baskets was set at 100 rpm. The dissolution system was combined with an automatic sampling station. Samples of 5 ml were withdrawn at 5, 10, 15, 20, 30, 45, 60, 80, 100 and 120 min. At was measured spectrophotometrically (Unicam Heλios Alpha, Spectronic Unicam, Cambridge, UK) at λ_{max} = 258 nm. Six parallel tests of dissolution were performed.

4.2.3.2. Preparation and investigation of PEP granules

The powder mixture containing 50% PEP and 50% MCC 101 was homogenized in a Turbula mixer for 10 min. The granules were prepared in a high-shear granulator (ProCepT 4M8 granulator, ProCepT nv, Zelzate, Belgium) with a glass bowl of 1000 ml. The granulation liquid was distilled water.

Chopper speed: 750 rpm
Impeller speed: 3000 rpm
Dosing speed: 4 ml/min
Spheronization time: 2.5 min
Total granulation time: 8.4 min

The average particle size analysis was carried out with an analytical sieve (Retsch GmbH, Haan, Germany). D50 was determined with sieving system software (Retsch EasySieve 2.0).

A dissolution tester with a paddle was used for the PEP content measurement and the dissolution study. The dissolution medium consisted of 900 ml of artifical gastric fluid (pH 1.2) kept at 37.0 ± 0.5 °C. The rotational speed of the baskets was set at 100 rpm. Samples of 5 ml were withdrawn at 5, 10, 15, 20, 30, 45 and 60 min. At was measured spectrophotometrically (Unicam Heλios Alpha, Spectronic Unicam, Cambridge, UK) at λ_{max} = 274 nm. Six parallel tests of dissolution were performed.

In the first step the 75 % PEP granules and 25 % tartaric acid were homogenized in the Turbula mixture for 10 min. The coated minitablet containing PAN (320 mg) and the PEP granules and tartaric acid mixture (120 mg) were filled into hydroxypropyl methylcellulose (HPMC) capsules which were placed into the basket of the dissolution tester. The PEP of dissolution from capsule was investigated in artificial gastric fluid (pH=1.2) after the PAN of dissolution was measured in phosphate buffer (pH=6.8).

5. RESULTS AND DISCUSSION

5.1. Investigation of the applicability of proteins as excipients

5.1.1. Investigation of film-forming effect of HSA

5.1.1.1. Yield

After the preparation of the intermediates, the yields were calculated. It can be seen that the yields of the samples containing HSA were very good (Table 5). The value for S0 was the lowest. The long treatment of this sample could cause a higher ratio of lost particles through the filter system. This was not detected for the other samples, which can be explained by the increase in their particle size, so that the possibility of loss was lower.

Table 5. Yield of preparation

Sample	Yield (%)
S0	73.04
S1	96.35
S2	94.26
S3	97.3
S4	94.52
S5	95.39

5.1.1.2. Particle shape

It can be seen from the SEM pictures that the surface of the original Arbocel A 300 was irregular (Fig. 2). The sticking of the treated particles is clearly visible, with the irregular

surface of the cellulose with binder layers of HSA forming bridges between the particles (Fig. 3). A covering layer (‘‘film-like’’) of HSA was detected at higher magnification. Numerous HSA spheres 1–2 μm in diameter were also produced during the process (Figs 4 and 5). This was a consequence of the spray-drying of the solution. These microparticles settled mainly in the irregularities in the powdered cellulose. Because of the size of these particles (smaller than the filter), the loss during the process can be relevant. The covering of the cellulose was very smooth, but a few defects could be detected. These can be due to the incorporation of the spray-dried particles into the layer, and the rupture of the HSA layer and the grained surface (because of the recrystallization of salts) (Figs 6 and 7).

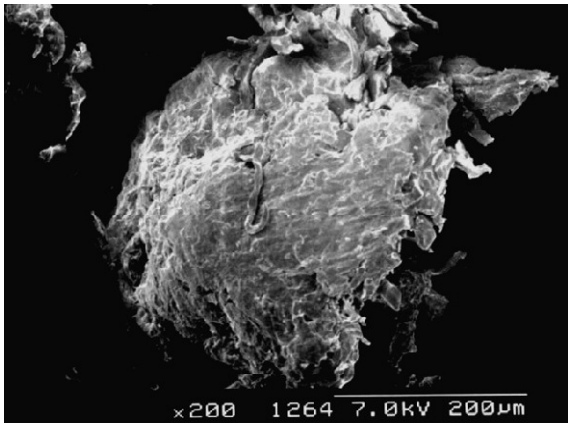

Fig. 2. Arbocel A 300 (SEM 200)

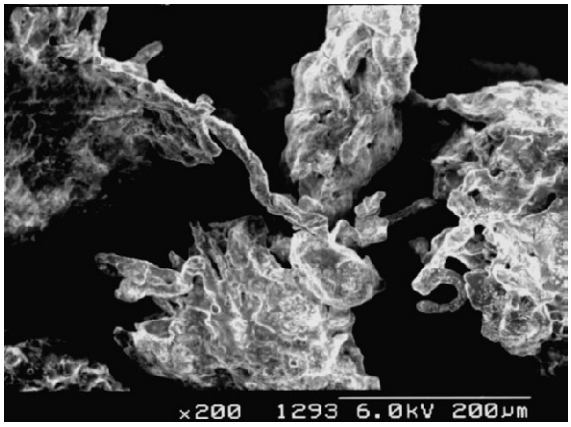

Fig. 3. S4 particle <315 μm (SEM 200)

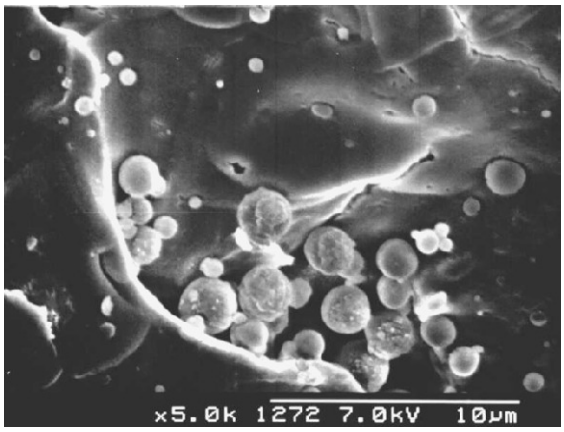

Fig. 4. Surface of S4 (SEM 5000)

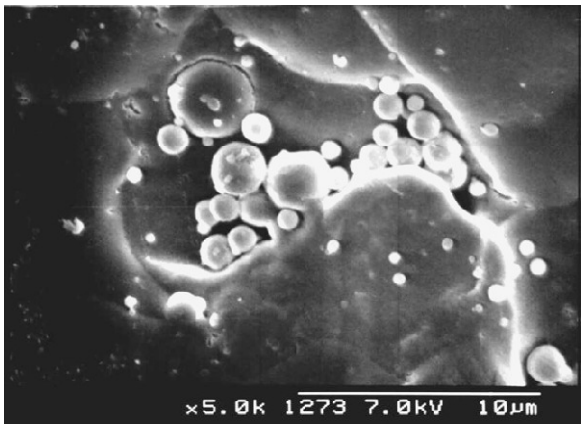

Fig. 5. Surface of S4 (SEM 5000)

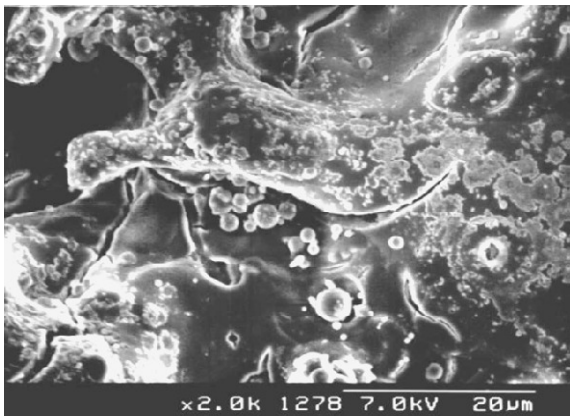

Fig. 6. Surface of S4 (SEM 2000)

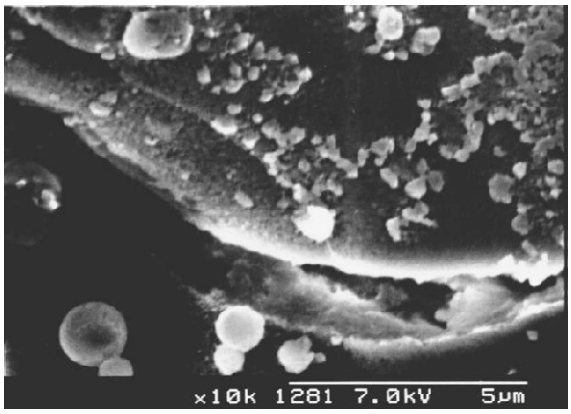

Fig. 7. Surface of S4 (SEM 10000)

5.1.1.3. Particle size analysis

The application of HSA caused an increase in the size of the particles (Table 6 and Fig 8). These results emphasized that HSA was a binder, since the coating could not cause an increase of more than 50% in the diameter. There was no obvious relationship between the concentration of the liquid and the particle size. This can be a result of the complexity of the process. It is known that the viscosity of HSA solution changes with the concentration [112]. At the lowest concentration, the spreading of the liquid on the solid surface is easier (because of the lower viscosity and density), so the covering and the sticking between particles were also higher. In this case, however, the processing time was longer and therefore the mechanical stress for these samples was higher, which could cause the abrasion of the products. Hence, an optimum concentration was necessary, where the increase in the aggregates was the lowest (the ratio of the two converse processes was the same). The lowest increase in D50 was detected for sample S4. Thus, this concentration was the most appropriate.

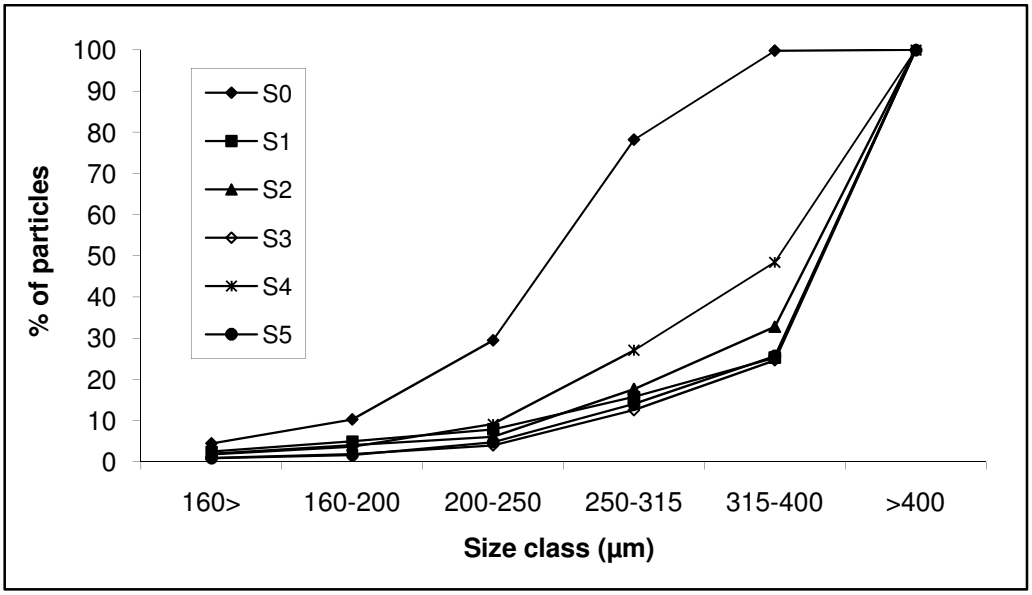

Fig. 8. Cumulative granulometric curve

The products were classified according to their particle size. During the fluidization process, the spray-drying of the atomized liquid is inevitable. These particles can be found among the particles smaller than 200 µm. The most appropriate particles measured between 200 and 315 µm; the highest proportion of starting materials is to be found in this range. Thus, the agglomeration is lowest for these particles. The aggregated products were larger than 315 µm. The proportion of the particles with optimum size for Arbocel A 300 was nearly 70%. The ratio of these particles was significantly lower for the samples containing HSA. The highest value was detected for S5, which was ~ 2 times higher than for the other samples.

5.1.1.4. Flowability

It is well known that the flowability of a sample is determined by the morphological parameters [113]. The flow time of the samples was therefore determined before sieving (Table 6). The flow time was long, with a high deviation, for the particles containing HSA. The best result was detected for S4. This product exhibited the lowest particle size increase. Because of the lower ratio of the irregular sticking of the particles, the shape was better for even flow.

Table 6. Properties of samples

Sample	D50 (µm)	<200 µm	200-315 µm	>315 µm	Flow time (s)
Arbocel A 300	277	10.3	67.9	21.8	13.4 ± 1.6
S0	258	21.3	67.0	12.7	7.7±0.2
S1	466	5.0	10.8	84.3	105.5 ± 26.0
S2	452	4.1	13.6	82.5	46.8 ± 7.9
S3	467	1.9	10.7	87.4	73.4 ± 10.9
S4	406	3.7	23.4	72.9	26.5 ± 5.5
S5	465	1.7	12.4	85.9	231.3 ± 29.1

5.1.1.5. Content uniformity

The study of the concentration of the active agent revealed that the concentration of HSA was higher in the group containing smaller particles (higher than the calculated value) (Table 7). This can be explained by the spray-drying of the active agent and the abrasion of these spheres from the larger particles (Figs 4 and 5). The lowest concentration was measured for S3, and in this case the proportion of these particles was also the lowest (Table 7). The formation of these particles was therefore the smallest in this case. Spray-drying is easier for liquids containing a high amount of solid, so the ratio of these particles should be enhanced on increase of the concentration of HSA. However, the larger density and hence the larger viscosity caused a decrease in the sprayability of the liquid. Larger droplets were formed, and thus the evaporation of the liquid took longer. In view of the adverse effects, the optimum value for the concentration of HSA must be found. In this case, 12.5% HSA was optimum. The sticking and the abrasion of the particles were explained previously.

The value for the largest particles could not be evaluated exactly. The determined value was lower than in the other two groups, but it was very variable for the same sample (8.75% was measured for S2, but repetition of this measurement gave >13%, with a high standard deviation (RSD >15%), i.e. the results of the repeated measurements were not comparable). This was influenced by the sampling from agglomerates containing particles with very different particle sizes.

In the fraction of particles in the interval 200–315 µm, the lowest value was measured for S4 (with the highest RSD). The HSA concentration of the small particles was also high. Accordingly, an uneven distribution of the active agent can be expected. The presence of the active agent could not be evaluated (loss of small particles, or enrichment in the larger

particles as a thicker film), because the exact determination of the concentration of the largest particles was impossible. In spite of its good flowability, S4 was not the best composition because of the uneven distribution of HSA.

Table 7. Concentration of HSA (%) in different intermediates

Sample	<200 µm	200-315 µm
S1	16.37±0.94	13.31±0.61
S2	19.10±0.88	12.63±1.25
S3	13.96±0.45	13.49±0.54
S4	18.68±0.83	10.95±1.36
S5	18.90±0.46	14.43±0.28

The main aim was to prepare intermediates for the preparation of tablets with different concentrations of HSA. It can be concluded that the yield of the fluidization technique was very good, independently of the concentration of the applied liquid. The process caused an increase in the particle size. The HSA was responsible for the increase, because water did not cause aggregation. The least increase was detected for the samples prepared with liquid containing 15% HSA (about 2 times higher than for the second best). In this case, the ratio of the particle size-increasing and destructive processes was the most preferable.

Not only the size, but also the shape of the particles changed, leading to a change in flowability. The concentration of HSA in the fraction containing smaller particles was higher because of the abrasion of the particles and the spray-drying of the HSA. The formation of large aggregates must be eliminated, because the distribution of the active agent is very inhomogeneous.

Finally, it may be stated that this modified layering of powder cellulose with a fluid bed technique can be applied for the production of an intermediate from HSA for the preparation of solid dosage forms. Previously, the appropriate concentration of this protein solution must be optimized, as HSA can act as a binder. Our results indicated that the best value can be reached with liquid containing between 12.5% (the most homogeneous distribution of HSA) and 15% HSA (the best flowability).

5.1.2. Investigation of granulation effect of HSA solution

5.1.2.1. Particle size

The yields of the samples containing MCC were very good (Table 8). The values for powdered cellulose (G9 and G10) were the lowest because large, very loose flakes were formed in these cases. The particles of samples produced with the well-known binder were smaller than those of G1. The particles of the samples containing HSA were larger than those of the samples containing the same powder mixture, but prepared only with water (G2, G6, G8 and G10). The conventionally used binder in the same concentration exhibited lower particle sizes than that of the sample prepared with HSA. The explanation may be the different wetting of the powder mixture and the different binding potency of the binder.

The surface tensions of the liquids, which can influence the wetting of the powder mixes, were 42.66 ± 0.62 Nm for HSA solution, 44.92 ± 0.94 Nm for HPMC solution and 40.26 ± 0.43 Nm for HPC solution. There was no relevant difference in this parameter, which was therefore not the main factor responsible for the particle size of the granules. The spreading of the liquid can be different because of variant viscosity (1.5 mPas for HSA solution, 19.1 mPas for HPMC solution and 45.9 mPas for HPC solution). The particle size was lower for granules prepared with viscous solution. The favourable granulating capacity of HSA solution can probably rather be explained by the better binding capacity of HSA solution.

For the evaluation of the effects of the starting materials, different parameters of the MCCs and liquids were considered. The particle sizes, bulk densities and Enslin numbers for the water uptakes of the different MCCs were determined. MCCs with higher bulk densities exhibited higher Enslin numbers (Table 9). A higher amount of fibres can bind a higher amount of liquid. When the water uptake was higher, the D50 value for the sample prepared with HSA solution was also higher (Table 8; G1, G5, G7 and G9).

Table 8. Properties of granules

Sample	Yield (%)	D10 (µm)	D50 (µm)	D90 (µm)
S1	99.21	607	885	1284
S2	91.85	111	608	1195
S3	87.16	114	687	1139
S4	87.06	188	301	904
S5	90.70	122	1054	1411
S6	84.95	68	334	743
S7	85.53	437	837	1194
S8	90.60	287	696	1130
S9	57.09	248	628	1288
S10	57.45	150	574	1272

The corresponding tendency was the opposite for the samples produced with water. The explanation may be that the higher amount of water taken up by MCC cannot act as a binder, since this material is not soluble. The only binder in this composition is the recrystallized MAN. The proportion of the water available as the solvent of MAN was therefore lower, and accordingly these granules were smaller. The liquid taken up by the MCC also contained HSA, for the samples were prepared with HSA solution. After drying, this component formed bridges between the particles, and in this case the most important binder was not the recrystallized MAN, but the HSA. When the amount of granulating fluid taken up was higher, there was a higher possibility of formation of more HSA bridgesthe , and hence the mean size of the samples was higher.

Table 9. Properties of MCC

Type	Particle size* (µm)	Bulk density * (g/cm^3)	Enslin number (ml/g)
101	50	0.29	2.91±0.05
103	50	0.32	3.03±0.06
105	25	0.23	2.45±0.12

* Data from the producer

5.1.2.2. Flowability

The properties of the granules were determined. Their flowability and compressibility were better than those of the starting powder mixtures (Table 10). The flow times for the samples with water (G2, G6, G8 and G10) were slightly lower than those for the samples containing binder. This difference was not of importance since these values are very good. The bulk densities of the samples prepared with HSA (G1, G5, G7 and G9) were higher than those of the granules prepared with water (except for G7 and G8, where there was no significant ($p < 0.05$) difference). This parameter was considerably lower for the samples prepared from powdered cellulose (G9 and S10) because they formed large fluffy flakes. The compressibility (Carr index) is another important parameter in tablet making. It was significantly better for the samples containing different binders. This can be explained by the better distribution of the particle size. HSA at this concentration caused a more appreciable increase than the conventional binders.

Table 10. Properties of granules and powders

Sample	Flow time (s)	Bulk density (g/cm^3)	Carr index (%)	Breaking force (N)
G1	8.0±0.12	0.706±0.011	4.87±0.44	2.64±0.29
G2	7.1±0.15*	0.639±0.006*	11.15±1.39*	2.43±0.55
G3	7.7±0.12	0.670±0.003*	7.69±0.77*	2.44±0.39
G4	6.3±0.12*	0.624±0.001*	7.44±0.44*	1.93±0.36*
G5	8.8±0.6	0.658±0.002	11.79±0.59	2.11±0.35
G6	6.5±0.12**	0.568±0.010**	14.10±0.44**	1.97±0.38
G7	7.9±0.15	0.714±0.007	4.62±0.77	2.70±0.63
G8	7.4±0.12**	0.723±0.006	8.53±0.62**	2.49±0.35
G9	10.2±0.21	0.258±0.005	6.67±0.44	2.36±0.41
G10	9.8±0.25	0.272±0.005**	9.23±3.35	2.28±0.5
MCC101+M	34.2±6.38	0.399±0.006	24.87±1.94	-
MCC103+M	31.9±2.2	0.434±0.0004	23.98±2.83	-
MCC105+M	No flow	0.455±0.012	28.66±2.16	-
PC+M	19.8±0.93	0.434±0.010	25.90±1.60	-

* Significant difference between the sample and G1.

** Significant difference between the samples prepared from the same powder mixture, but with a different granulating fluid.

5.1.2.3. Mechanical properties

The mechanical properties of the granules were better for S1 than for the compositions containing the conventional binders. Higher values were found for the samples containing HSA (G1, G5, G7 and G9), independently of the type of cellulose. The best mechanical properties were those of the samples containing MCC 105 (G7 and G8). The water uptakes of these samples were lowest because of the low density (Table 9). The number of fibres and thus the number of bridges formed may be lower, but the binding may be stronger and accordingly the hardness may be higher. The higher possibility of the binding force of recrystallized MAN must also be considered. The texture of this sample was the most compacted.

Not only the breaking hardness, but also the deformation process can provide information on the processibility. The breaking curve of S1 (Fig 9) was very similar to those of the compacted pellets or granules [114].

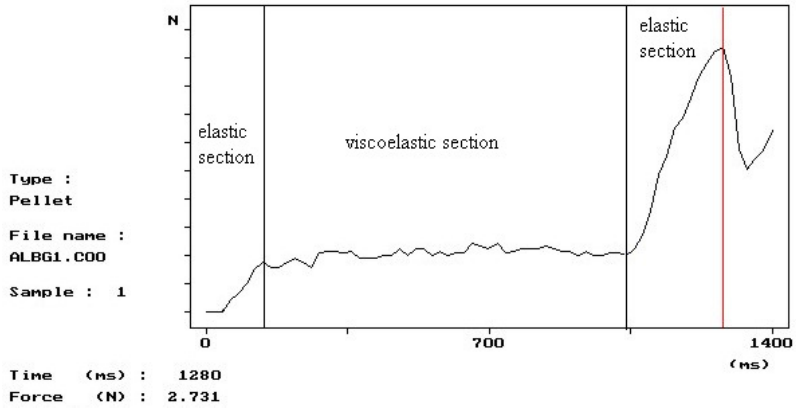

Fig. 9. Breaking hardness curve of sample G1 (granulating fluid: HSA solution)

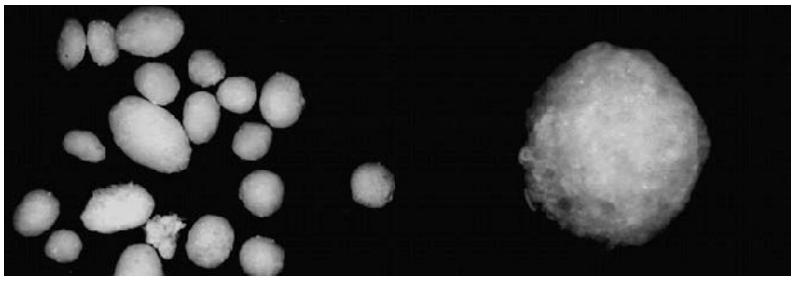

Fig. 10. G1 granules (magnification: 10x (left) and 50x (right))

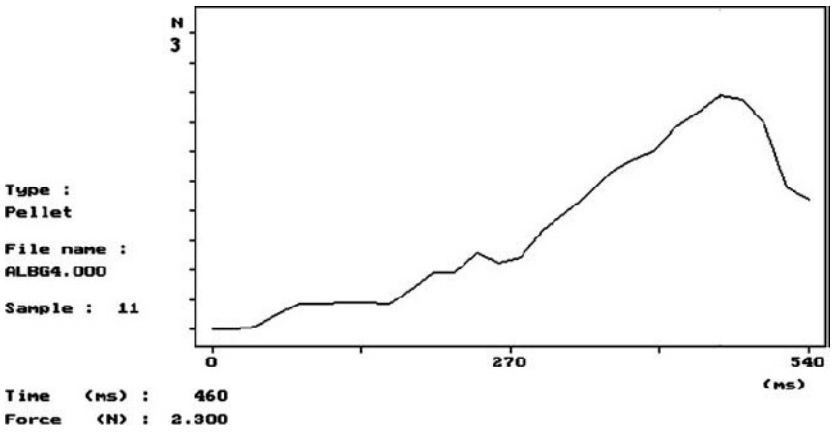

Fig. 11. Breaking hardness curve of sample G2 (granulating fluid: water)

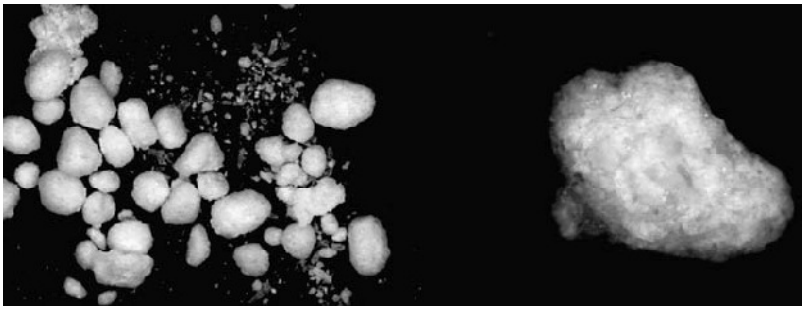

Fig. 12. G2 granules (magnification: 10x (left) and 50x (right))

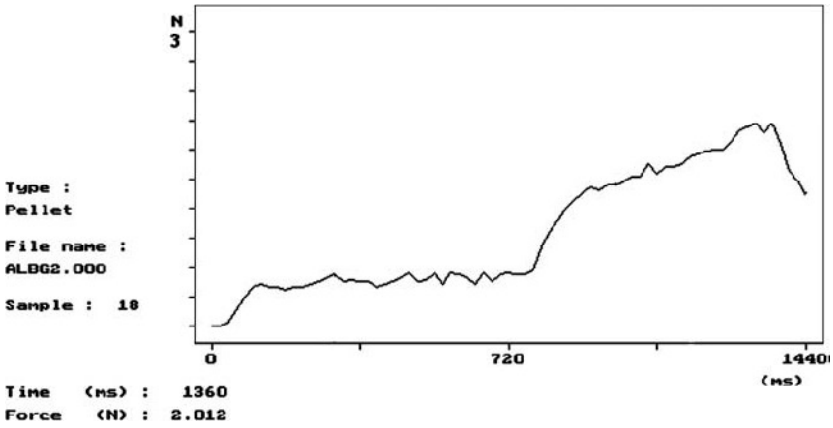

Fig. 13. Breaking hardness curve of sample G3 (granulating fluid: HPMC solution)

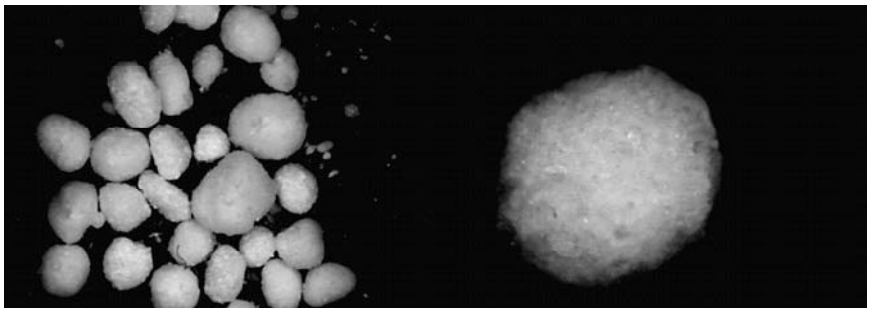

Fig. 14. G3 granules (magnification: 10x (left) and 50x (right))

There were three phases: a short elastic part was followed by a viscoelastic phase, and finally an elastic section up to the breaking point (Fig. 9). There were no meaningful irregularities in the curves, which revealed only small deformations caused by the slightly inhomogeneous structure (Fig. 10). G2, prepared with water, exhibited a primarily elastic curve with a short viscoelastic section (Fig. 11). It is well known that air exhibits elastic properties and in this case the amount of entrapped air was higher because of the loose structure and irregular shape (Fig. 12). More elastic materials cannot be compressed because of the capping [36]. The curve of G3 (Fig. 13) was better than that of G2, but there were more irregularities than for G1, and the separation of the different phases was also less marked. The shape of these particles was very similar to that of G1 particles (Fig. 14).

It can be concluded that the HSA solution had a very good granulating effect when the system contained the studied MCCs. Powdered cellulose in this composition was not appropriate for granulation with HSA solution. As compared with the conventionally used binder in the same concentration, the granules formed with HSA displayed a larger particle size, a significantly better compressibility, a higher breaking hardness and a favourable deformation process. The explanation of the advantageous properties is the good adhesive properties of the protein. The different MCCs furnished products with different properties. This was connected with the structure of the particles and their water uptake. According to our results, the best compressibility (highest bulk density), and the best mechanical behaviour were detected for materials containing MCC 105 and granulated with HSA solution.

It may be stated that the inclusion of HSA in the granulating fluid can be very useful: pretreatment of this component is not necessary, the granulating effect is considerable and the amounts of additives can be decreased.

5.2. Evaluation of the applicability of proteins as active agents

5.2.1. Investigation of enzyme activity

5.2.1.1. Effect of direct compression

In the first step, the effect of the compression force on the enzyme activity of PAN and PEP was determined. There was no significant alteration in this parameter for PAN, a slight decrease in enzyme activity was detected for PEP acid mixtures at high pressure. The application of low compression force is therefore proposed for these compositions (Table 11). The heat generated during compression can induce the degradation of this active component. Its incidence and extent can depend on the tablet-making parameters and on the properties of the tablets. Its direct study is very difficult. To investigate the effects of elevated temperature on the enzyme activity of PAN and PEP, independent thermal-stress tests were performed. The untreated PAN and PEP were stored for 2 h under dry air conditions in a thermostat (Hereaus Instruments, Hanau, Germany) in which the heat was distributed homogeneously. No significant effect was observed (except for PEP at high temperature), and it can therefore be concluded that direct compression can be applied for the processing of PAN, since neither the pressure nor the elevated temperature in the "hot spots" induced any appreciable modification of the function (Table 12). PEP and its mixture with acids can also be processed with direct compression, but lower pressure must be applied.

Table 11. Starch-hydrolysing activity of PAN treated by compression

Load (t)	**Activity of PAN (%)**	**Activity of PEP**		
		100% PEP	**50% tartaric acid**	**50% citric acid**
2	98.87±0.03	101.73±3.48	99.20±3.05	102.36±0.71
4	98.84±0.13	101.97±1.45	99.75±3.73	100.54±3.18
6	98.61±0.09	102.05±2.63	88.51±4.94	97.69±0.55
8	98.52±0.19	98.72±3.21	91.67±2.80	96.90±2.88
10	98.61±0.11	99.20±3.01	89.14±2.20	95.63±3.16

Table 12. Starch-hydrolysing activity of PAN treated by elevated temperature

Temp. (°C)	Activity of PAN(%)	Activity of PEP (%)
40	100.01±0.04	99.35±3.38
50	99.62±0.18	-
60	99.54±0.02	99.91±2.24
70	99.69±0.08	-
80	99.65±0.09	96.43±2.07
100	99.60±0.13	91.75±4.46

5.2.1.2. Effect of the wet granulation

Close to 100% enzyme activity of PAN was detected for some of the samples treated with water at low temperature, whereas the highest value was nearly 70% for the samples wetted with ethanol. It is also to be seen for both liquids that special combinations of factors can induce an enzyme activity of PAN of only 7% (Table 13).

Table 13. Starch-hydrolysing activity of PAN treated by wetting

Temp. (°C)	Time (h)	Content of liquid (%)	Activity - water (%)	Activity - ethanol (%)
40	1	30	99.04±0.23	47.28±2.23
40	1	60	98.61±0.01	67.55±2.67
40	2	30	98.98±0.15	19.15±4.08
40	2	60	84.44±1.53	30.62±0.78
60	1	30	23.63±3.31	7.07±2.09
60	1	60	6.91±3.02	7.9±0.92
60	2	30	11.08±1.83	8.85±2.71
60	2	60	9.17±1.71	7.65±3.25
50	1.5	45	28.11±2.81	15.84±3.6
50	1.5	45	26.78±2.48	11.65±1.08

The enzyme activity of PEP was nearly 100% without the application of acids at low temperature, water content and within a short time. In this condition with tartaric acid the activity was near 100%, but the citric acid induced a relevant decrease. It is also to be seen for

both acids that special combinations of factors can induce the nearly total loss of PEP activity (Table 14).

Table 14 Relative activity of PEP

Factors			PEP activity (%)		
Temp. (°C)	**Time (h)**	**Content of liquid (%)**	**100% PEP**	**50% tartaric acid**	**50% citric acid**
40	1	10	104.87±3.8	101.08±5.27	73.71±4.77
40	1	30	93.34±4.07	98.41±4.07	90.12±3.10
40	2	10	103.26±7.99	87.02±1.82	63.69±1.20
40	2	30	103.11±2.62	89.76±4.18	73.83±2.90
60	1	10	102.19±5.08	14.18±0.45	1.45±1.47
60	1	30	92.63±1.55	16.14±0.35	1.42±0.26
60	2	10	97.71±2.55	2.08±0.13	0.39±0.48
60	2	30	94.34±4.24	0.51±0.46	1.03±0.50
50	1.5	20	95.53±3.61	58.99±2.53	22.17±0.35
50	1.5	20	98.94±3.08	45.69±1.44	18.87±0.93

For the exact evaluation of the effects of the factors, linear fitting was applied in all cases (Table 15). The b_0 value was ~54 % lower for ethanol than for water for PAN. In the case of untreated PEP, b_0 was 98 and with acids was lower. Since this is a mean value, it can be concluded that in all cases PAN is more sensitive to ethanol than to water, and in all cases of PEP is more sensitive to citric acid than to tartaric acid. Only temperature was a significant factor for both liquids and for both acids. In this case, the other factors did not cause significant changes. High temperature did not cause a decrease in the starch-hydrolysing activity of relatively dry PAN (untreated PAN with a ~7% moisture content). Conventional granulation/pelletization can therefore induce a decrease in the enzyme activity of PAN and PEP, when elevated temperature and high liquid content are applied at the same time. The other explanation for the importance of this step is the fact that topical overwetting (a higher amount of liquid can be detected in certain microvolumes of the mass) cannot be avoided during the wetting in the granulator.

Table 15. Effects of factors

Factor	Coefficient for water for PAN	Coefficient for ethanol for PAN	Coefficient 100% PEP	Coefficient 50% t. a.	Coefficient 50% c. a.
R^2	0.9253	0.9481	0.944	0.994	0.957
b_0	48.67	22.36	98.59	51.38	34.67
b_1 (temperature)	-41.29*	-16.64*	-4.43	-85.84*	-74.26*
b_2 (liquid content)	-4.2	3.92	1.35	-12.61	-6.94
b_3 (time)	-3.06	-7.95	-6.15	0.11	6.79
b_{12}	-0.46	-4.02	-2.73	-1.25	6.21
b_{13}	0.49	8.32	-0.31	0.08	-6.48
b_{23}	0.09	-1.35	4.39	0.47	-1.40
b_{123}	3.62	0.85	-1.30	-2.23	1.73

* significant ($p<0.05$)

t. a.: tartaric acid

c. a.: citric acid

Time and its interaction with temperature exhibited non-significant, but considerable effects for ethanol (b_{13}=8.32). The two factor interactions were irrelevant in the other cases.

It can be concluded that the enzyme activity of PAN and PEP was not changed because of direct compression. High temperature did not induce a decrease in this parameter for the relatively dry active component. Modelling of the wet conditions which can occur during granulation and coating led to significant modifications. Ethanol caused more relevant changes than water for PAN, thus this component must be avoided during the formulation. In both cases, the most important factor was temperature. The PEP activity was altered significantly with application of acids. The citric acid caused a more relevant decrease.

This information is helpful as concerns the design of the preparation of multiparticulate dosage forms containing PAN and PEP. Wet granulation can be applied for the processing of PAN if the temperature is < 40 °C, only a low amount of water is applied, and the process time is short. A better way is to produce minitablets by direct compression, since degradation of the active agent can then be avoided. Wet conditions must also be considered during the coating. This step is obligatory in this case and ethanol cannot be used as liquid. Aqueous systems must be applied at low temperature, but quick drying is also advisable, since

overwetting of the surface can occur. The additional operational parameters applied during the surface treatment must therefore be optimized for the quick drying of the first layer of the film (e.g. using slow atomization with a high amount of drying air in the first phase, and later the rate of atomization can be enhanced). Such preformulation studies can reveal the importance of the different parameters, and are necessary for optimization of the preparation of dosage forms containing sensitive proteins or enzymes.

PEP can be processed with wet granulation without the application of acids or with tartaric acid at low temperature and within a short time. The PEP activity decreased significantly with increasing temperature in case of citric acid. The processing of PEP with citric acid is not recommended with wet granulation. This study emphasizes the importance of special aspects in the process of solid dosage forms containing enzymes.

5.2.2. Investigation of properties of surface

5.2.2.1. Study of spreading of coating liquid on tablets containing PAN

It is well known that numerous problems can arise if the spreading of the coating liquid on the core is inadequate. The wetting of pure PAN was very poor, so it is not the most appropriate for coating (Table 16).

Table 16. Contact angles on tablets containing different ratios of PAN and MCC

PAN (%)	**Without Mg-st (°)**	**With Mg-st (°)**
0	29.5±1.7	40.1±2.1
10	36.3±1.0	46.5±1.8
20	44.1±1.7	51.5±1.6
30	50.4±1.6	58.4±1.9
40	58.2±1.2	64.5±2.1
50	65.9±1.3	69.5±2.3
60	67.6±1.5	72.9±2.1
80	72.5±1.8	76.2±1.9
100	74.6±1.9	80.4±1.4

In case of the direct compression the conventionally used binder is MCC, which exhibited significantly lower contact angles. Contact angles of the coating liquid on comprimates prepared from different mixtures of these components were evaluated and the

application of hydrophobic Mg st. (contact angle: 92.7 ± 2.2°) was also used in this evaluation. It significantly ($p < 0.05$) increased the contact angle for every composition.

The change in the contact angle was evaluated in correlation with the concentration of the active agent. The correlation of linear fitting (R2) was 0.9099 for mixtures without Mg st., and 0.9344 for those with Mg st. (Fig. 15). Thus, it can be concluded that the tendency was not linear; as a linear change was expected, further investigation of the mixtures was necessary. The theoretical values of the contact angle were determined from the results on the pure materials, and the differences between the theoretical and measured values were studied. Linear fitting between the parameters of the starting components was applied for the determination of the theoretical contact angle of the mixtures. This calculation was performed for the samples with and without Mg st. The absolute and relative deviations are presented in Table 17. Since the signs of these values were positive, the relevance of the more hydrophobic material can be detected. This phenomenon is undesirable for the coating. In both cases, the maximum difference was detected at 50%. The relative change was more than 25% for the sample prepared without lubricant. The application of Mg st. led to decreased differences from the predicted values.

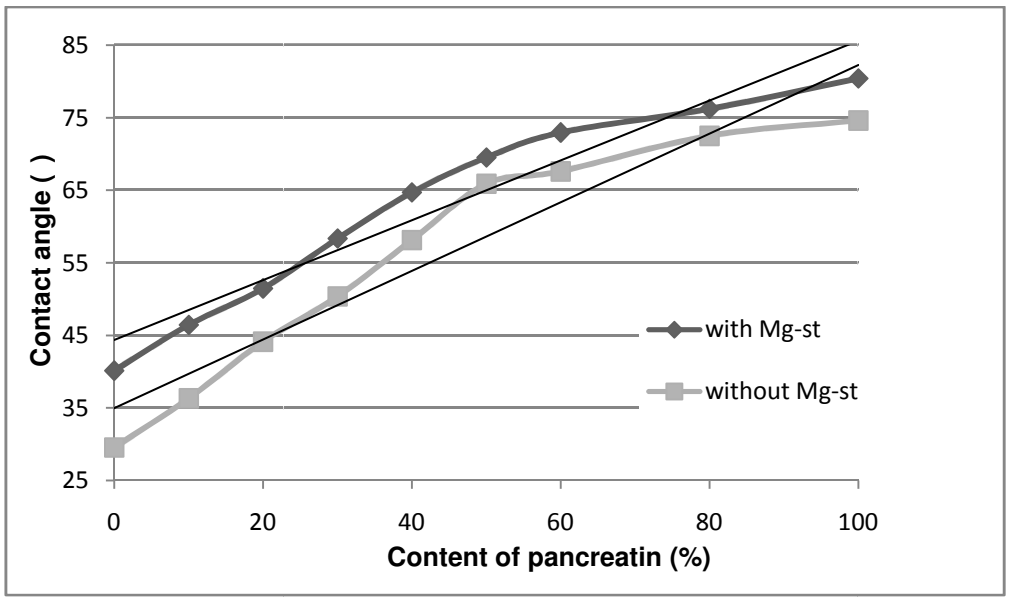

Fig. 15. Contact angles for mixtures without Mg st. and with Mg st.

Table 17. Deviation of measured contact angle from the theoretical value

P	without Mg st.		with Mg st.	
	(°)	(%)	(°)	(%)
0	0	0	0	0
10	2.3	6.7	2.3	5.2
20	5.6	14.5	3.3	6.8
30	7.3	17	6.2	11.8
40	10.6	22.2	8.5	15.1
50	13.8	26.6	9.3	15.3
60	11	19.5	8.7	13.5
80	6.9	10.5	3.9	5.4
100	0	0	0	0

In this study, the effect of the surface free energy was investigated. This parameter was calculated from the wettability of the components. The lowest surface free energy was detected for Mg st., and the highest for MCC (Table 18).

Table 18. Wettability and surface free energy of components

Sample	Θ_{water} (°)	$\Theta_{diiodomethane}$ (°)	γ^d (mN/m)	γ^p (mN/m)	γ^{tot} (mN/m)
PAN	36.34±1.97	51.76±1.85	30.65	32.84	63.49
MCC	25.98±1.96	15.09±1.92	44.27	32.15	76.42
Mg st.	96.07±1.87	62.38±1.87	26.77	4.37	31.14

The spreading coefficient was calculated from these data. When the sign of the spreading coefficient (S_{12}) is positive, material 1 spreads on the surface of material 2. Every combination of the components was tested, and the positive cases are listed in Table 19. It can be seen that PAN covered the MCC particles. Since the wettability of pure PAN is poorer than that for MCC, this arrangement explained the higher measured contact angle than expected from the theoretical value. Mg st. covered both of the other components. The spreading coefficient was higher for MCC. Since Mg st. covered both components, the difference between the theoretical and measured values was decreased.

Table 19. Spreading coefficient (mN/m)

Material 1	**Material 2**	**S_{12}**
PAN	MCC	10.4
Mg st.	MCC	19.8
Mg st.	PAN	10.3

5.2.2.2. Friction and plasticity studies

The R, PLS-M and FW values were calculated for both main components and for mixtures containing 50% PAN (Table 20). The highest FW and lowest R were detected for MCC, but PLS-M was highest for this component. PAN exhibited better surface properties (lower FW) and poorer PLS-M. For the binary mixture, PLS-M, which describes the bulk property, was nearly the same as the calculated value (the average of the data for the pure materials). The parameters relating to the surface properties were also closer to the values for PAN. This was in accord with the findings of the wettability study.

Table 20. Compactibility parameters

	R	**PL_{S-M} (%)**	**FW (Nm)**
MCC	0.89±0.003	80.8±0.64	0.296±0.019
PAN	0.92±0.002	77.9±1.15	0.142±0.015
MCC+PAN	0.93±0.002	79.1±0.65	0.153±0.011
MCC+PAN+Mg-st.	0.94±0.004	78.2±1.30	0.150±0.016

The properties of powder mixtures with and without Mg st. exhibited very similar behaviour. Slightly better R values were measured for the samples containing Mg st. A significant effect was observed in the wettability of these powder mixtures, but this phenomenon was not detected in the compactibility parameters.

It can be stated, that the extent of spreading of the coating liquid to form an enteric soluble film on the tablets decreased with increasing amount of PAN. However, the change in this parameter was not proportional to the concentration of this component. The deviation from linearity was highest for the mixture containing 50% P. A deviation was also detected in the presence of 1% Mg st., but its extent was then lower. The enrichment of the PAN on the surface of tablets prepared from binary mixtures was supported by the spreading coefficient between the components. The spreading coefficient of Mg st. revealed that both components

were covered by this lubricant. The deviation from the predicted wettability was therefore decreased when this excipient was applied.

The plasticity was not affected in a similar way as the wettability, for it is a bulk property. The alteration in the friction exhibited a similar tendency to the spreading of the coating liquid on tablets containing powder mixtures. Thus, a change in composition caused more appreciable alterations in the properties of the surface of the tablets than in the tablettability. Mg st. caused less change in the friction than in the wettability.

The determination of surface free energy can be a useful tool for prediction of the microstructure of the surface of tablets.

5.2.3. Formulation of solid dosage form containing protein

Based on the preliminary results a complex multiparticulate system can be formulated from enteric coated minitablets coantining PAN and granules containing PEP and tartaric acid.

5.2.3.1. Preformulation study

It can be seen that the flow properties and the compressibility of PAN and the powder mixture were not the most suitable for tabletting, but the mixture is processable on a lab scale (further optimization is needed).

Table 21. Results of preformulation studies

	PAN	**Powder mixture**
Hausner factor	1.45±0.01	1.39±0.01
Carr index (%)	31.0±0.4	28.3±0.4
Moisture content (%)	6.68	5.92
Flow time (s)	60.4±5.96	54.3±7.16
Average particle size (μm)	134	-

5.2.3.2. Properties of minitablets

The disintegration time of the PAN minitablet without a coat in distilled water was 4.99 ± 0.55 min (Table 22). In the case of the coated tablet disintegration could not be observed in the artificial gastric fluid (pH=1.2) for 2 h, but it disintegrated during 13.65 ± 2.0 min in phosphate buffer (pH=6.8), so it complies with Ph. Eur. requirement. The measured content of

PAN accords with the calculated content of PAN (without coat: 50 %, coated: 41.72 %). The other phisycal parameters were also in accordance with Ph. Eur. The activity of PAN was not decreased significantly either after compression or after coating.

Table 22. The parameters of PAN minitablets

Parameters	Without coat	Coated
Mass (mg)	18.3±0.7	24.6±0.9
Diameter (mm)	3.27±0.02	3.36± 0.05
Height (mm)	2.01±0.25	2.32±0.14
Content of PAN (%)	49.75±1.63	38.42±1.54
Disintegration pH=1.2 (min)	13.65±2.0	>120
Disintegration pH=6.8 (min)	-	4.99±0.55
Breaking force (N)	55.62±3.37	-
Friability (%)	0.94	-
Activity of PAN (%)	97.91±0.31	96.68±0.30

In the first step, the dissolution was tested in artificial gastric fluid (pH=1.2). In this case, the liberation of PAN was not observed for 2 h. After 2 h, the dissolution medium was changed to phosphate buffer (pH=6.8). In this medium the amount of dissolved drug at 100 min was 100%. In the first 15 min, the dissolution was slow (Fig. 16), since the coating was not dissolved completely, but, after this lag time the dissolution of the coating was complete and fast dissolution of the PAN began.

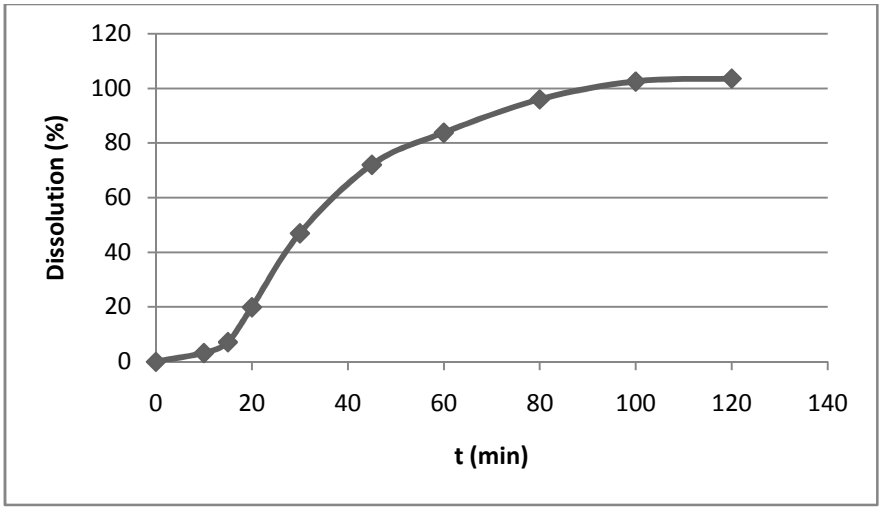

Fig. 16. Dissolution of PAN in phosphate buffer (pH=6.8)

5.2.3.3. Investigation of PEP granules

Based on the preliminary results PEP tartaric acid mixture was selected, and a high shear granulation under moderate circumstances were applied. The average particle size (D50) of granules was 578 μm. 51.4 % of the particles were in the range 315-630 μm; in the case of tartaric acid, it was 80%. These fractions were used for filling into capsules. The D50 of tartaric acid was 400 μm. The PEP content was 49.33%, which correlates with the calculated value (50%). The amount of dissolved drug at 5 min was 100%.

5.2.3.4. Preparation of final dosage form

The multiparticulate solid dosage form containing PEP and PAN was prepared with filling of minitablets and granules into the same capsule. The previous results relating to the enzyme activity were taken into consideration in the selection of the applicable pharmaceutical technology process. The activity of the enzymes was not decreased significantly and therefore these pharmaceutical technological processes are appropriate for the preparation of solid dosage forms containing PAN and PEP. Further optimization of the other factors was not performed in this study, but these should be taken into consideration during the formulation.

6. **SUMMARY**

The main aim of this work was to examine the applicability of the proteins during the processing into solid dosage forms.

Major conclusions for protein used as a pharmaceutical excipient:

- HSA solution can be applied in modified layering technique, since yield of the fluidization technique was very good, independently of the concentration of the applied HSA solution.
- The process caused an increase in the particle size. The HSA was responsible for the increase, because water did not cause aggregation.
- HSA solution had a very good granulating effect in the tested system. As compared with the conventionally used binder at the same concentration, the granules formed had a larger particle size, significantly better compressibility and higher breaking hardness.

- The advantageous properties are not reflected by a changed surface tension. The explanation for this was the good adhesive properties of protein.
- It may be stated that the application of HSA in the granulating fluid can be very useful, because pretreatment of this component is not necessary and the granulating effect is also considerable.

Major conclusion for protein used as active agent:

- Direct compression can be applied for the processing of PAN, since neither the pressure nor the temperature induced any appreciable modification of its function.
- Granulation with an aqueous system can be applied under well controlled circumstances (low moisture content, short process).
- In a conventionally used direct compression composition the enrichment of PAN on the surface of the tablet must be taken into consideration int he course of processing. It can be critical due to the wettability and water sensitivity of the API.
- To achieve the appropriate activity PEP must be applied in combination with acid. The combinations with citric and tartaric acid were appropriate for direct compression at low compression force.
- At low temperature, small amount of water and short process time, the activity PEP did not alter significantly for the sample with tartaric acid, and the wet granulation can therefore be used under these circumstances
- The processing of pepsin with citric acid is not recommended with wet granulation.
- Based on these preformulation tests, the production of intermediate products with suitable properties was feasible, the predicted importance of the factors were proven.

Practical usefulness

The importance of proteins in therapy is constantly increasing, thus formulation must support this tendency. The use of alternative administration routes for these active agents is also a reasonable demand of the patients. One option can be the use of the methods generally used for the solid dosage forms of small molecules. These methods were evaluated in this work and the following practically useful conclusions can be drawn.

- Some proteins have special properties to enhance the formulation. Main advantage of these properties, that the number of excipients and/or preliminary formulation steps

can be reduced. This is very important because the proteins are very sensitive materials so the number of additives must minimised.

- The modified layering technique in the fluid bed apparatus and the high shear granulation can be appropriate for the preparation of intermediate products of solid dosage forms containing proteins. Direct compression can also be applied for some proteins.

- The individual steps of these technologies must be understood and the critical control points must be appropriately controlled. PAT is currently becoming more and more important in the pharmaceutical industry during the manufacturing of medicines. In this study, some examples are demonstrated that can be critical control points in the course of the production of solid dosage forms containing proteins.

Considering these aspects is more important for sensitive drugs. The final conclusions of this study underline of this statement, and these results also show that the conventionally used equipments and methods (with some small modifications) can be applied for the formulation of solid dosage forms from certain proteins. The further evaluation of these aspects can promote the development of dosage forms for future therapies with proteins.

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ANNEX

I.

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Research paper

Formulation of an intermediate product from human serum albumin for the production of a solid dosage form

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Abstract

The main objective of this study was to evaluate and to increase the processibility of a model protein (human serum albumin (HSA)) for preparation of an intermediate for a solid dosage form. The applicability of the solid forms is easier, and therefore their formulation is a promising method for the application of proteins. The layering of powdered cellulose with HSA solutions of different concentrations in a fluid bed apparatus with the top spray method was applied. The yield of this technique was very good, independently of the concentration of the applied solution. The HSA covered the particles (the HSA layer formed was smooth), but it caused aggregation of the cellulose particles, and spray-dried microparticles also formed. The proportion of optimum-sized particles (200–315 μm) decreased. The largest amount was detected for the samples prepared with liquid containing 15% HSA (about 2 times higher than the second best). Not only the size, but also the shape of the particles was changed. The alteration in this parameter caused a change in the flowability. This was likewise the best for the samples prepared with the liquid containing 15% HSA. The concentration of HSA in the fraction containing smaller particles was higher, because of the abrasion of the particles and the enrichment of the spray-dried HSA. The distribution of HSA in the large particles was uneven. The layering of powder cellulose can be applied to produce an intermediate from HSA for solid dosage forms, but the appropriate concentration of this protein solution must be optimized previously because HSA can act as a binder. The formation of large agglomerates must be eliminated, because the distribution of the active agent in these is very inhomogeneous. The present results indicated that the best value can be achieved with liquid containing between 12.5% (most homogeneous distribution of HSA) and 15% HSA (best flowability).

Keywords: Human serum albumin; Fluidized bed technology; Layering; Powdered cellulose; Solid dosage form

1. Introduction

Biologically active peptides and proteins are increasingly becoming a very important class of therapeutic agents because of their extremely specific activity and high tolerability by the human organism [1]. Their rapid clearance in the body necessitates repeated injections, which is an inconvenient form of therapy, as well as being painful [2]. It is therefore reasonable to formulate dosage forms which can be applied by the patient without pain. Alternative routes for the systemic effect are currently becoming widespread. Thus, transdermal, rectal, nasal and buccal therapeutic systems can be used without the destructive effects of the gastrointestinal tract on the proteins [3–5]. The dosage forms can be liquid, semisolid or solid (tablets or capsules) with an appropriate bioadhesive effect [6–8]. The applicability of these solids (e.g., oral, tablets for buccal or sublingual use) is easier, and therefore their formulation is a promising method, though with many challenges.

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One possibility for tablet-making is direct compression [9,10]. The preparation of appropriate materials from the component present in a liquid (e.g., serum) for this method is very difficult because there are many important parameters (e.g., good flowability and compressibility) which must be considered [11]. Another problem with direct compression is the higher compressing force, and so higher temperature during the compression can be reached which can destroy the proteins. The larger particles of protein can arrange into "hot spots", where the temperature is higher [12–14]. However, the processibility of small particles is very difficult because of the autoadhesion [15,16]. The preparation of appropriate intermediates can promote the preparation.

The intermediate products can be different granules or particles prepared by the layering technique. This layering method is nowadays becoming more popular for the preparation of multiparticulate dosage forms, because conventional granulators (fluidized bed, centrifugal, etc.) can be applied and the small amount of active ingredient can be processed within a short time [17–19]. This technique conventionally uses an inert spherical carrier (e.g., sucrose or cellulose) with the same diameter. The compressibility of these spheres (non-pareils) is not appropriate, and it cannot be relevantly changed with other additives. Accordingly, other carriers must be used for the tablet-making. Powdered cellulose can be a promising component because it is inert and compressible with other cellulose derivatives, but the surface of this component is uneven.

The main objective of our study was to evaluate and to increase the processibility of model protein (human serum albumin (HSA)) in order to prepare an intermediate for an oral solid dosage form. HSA is a single-chain protein synthesized and secreted from the liver cells. HSA (M_w 66,472 Da) is found mainly in plasma (~50%) where it maintains the pH and osmotic pressure and has a major role in transporting a wide range of molecules such as metals, fatty acids, amino acids, metabolites and many drugs (e.g., interferon) [20–22]. It is known that HSA is sensitive to heat, high shear mixing and ions, etc. [23–25]. The choice of appropriate formulation (additives and methods) is therefore very difficult. A method was used which is appropriate for scaling-up. One of the available options was the HSA coating of a carrier in a fluid bed apparatus with the top spray method. Since there is no incompatibility between cellulose and HSA, powdered cellulose was used. This material is often utilized as a functional filler for tablet-making [26]. Many aspects must be considered during fluidization. In this part of our work, the concentration of the liquid was chosen as an evaluated factor. The information on this parameter is necessary to optimize the process of converting HSA into a solid dosage form and to determine the critical control points. The aim was to prepare a suitably flowing, even-covered intermediate with minimum agglomeration formation. The flowability and particle size of the products were therefore evaluated, and they were classified into three groups according to their size. The contents of active agent in the different groups were also tested.

2. Materials and methods

2.1. Materials

A solution containing 39.9 g/l of HSA (and also NaCl, KCl, Na_2HPO_4, KH_2PO_4, etc.) was used in this work (Trigon Biotechnological Ltd, Budapest, Hungary). Parameters of liquid were as follows:

Content of albumin: min. 98% of the total protein
Content of endotoxin: 5 IE/mg
Content of chloride: 4.85–5.35 mg/ml
Osmolarity: 285.0–315.0 mOsmol/kg

This liquid was lyophilized at −50 °C and 180–200 mTorr with a freeze-drying machine (Flexi-Dry MP FTS Systems, Inc., Stone Ridge, USA). The lyophilized products were dissolved in the original liquid (39.9 g/l) to produce liquids with contents of 5%, 10%, 12.5% 15% and 20%. The carrier was powdered cellulose with an excellent flowability and superior stability (Arbocel A 300, JRS GmbH&Co, Rosenberg, Germany).

2.2. Preparation of samples

The samples were prepared with a fluid bed apparatus (Strea-1, Niro-Aeromatic AG., Bubendorf, Switzerland). The top spray method was used. The concentration of the layering liquid was varied (see above). The quantity of HSA applied was the same, and several other factors were also varied for the sample. First, 100 g of powdered cellulose was treated with 300 g of water to evaluate the effect of water on the aggregation. It is known that the average denaturation temperature of HSA is about 60 °C so the drying temperature was lower [25]. The constant parameters were as follows:

Quantity of carrier: 100 g
Nozzle diameter: 0.8 mm
Drying temperature: 35 °C
Flow rate: 4 ml/min
Atomizing pressure: 2 bar
Blow-out pressure: 4.5 bar
Air volume: 40 m^3/h
RH of the input air: 45%
Preheating: 8 min without atomizing air
Process: in blocks (10-min atomization + 2-min drying)
Last drying: 8 min without atomizing air
The varied factors are listed in Table 1.

2.3. Morphological study

A Hitachi S2400 (Hitachi Scientific Instruments Ltd, Tokyo, Japan) scanning electron microscope was used to determine the shape and the surface of the particles. A sputter coating apparatus, Polaron E5100 (Polaron Equipment

Table 1
Parameters in preparation of samples

Sample	Concentration of HSA solution (%)	Quantity of the liquid (g)	Total process time (min)
S0	0	300	134
S1	5	300	130
S2	10	150	65
S3	12.5	120	50
S4	15	100	47
S5	20	75	33

Ltd, Hertfordshire, England), was applied to induce electric conductivity on the surface of the sample. The air pressure was 1.3–13 mPa.

2.4. Particle size distribution

The sizes and the size distributions of the samples were evaluated. An analytical sieve (Retsch GmbH, Haan, Germany) was used. D50 was determined with sieving system software (Retsch EasySieve 2.0).

Depending on the particle size, the products were divided into three groups (<200, 200–315 and >315 μm).

2.5. Flow properties

A powder testing apparatus (PTG-1, Pharma Test GmbH, Hainburg, Germany) was used to test the flow time of 100 ml of samples. A teflon accessory 10 mm in diameter and stirring at 25 rpm was applied. Three parallel experiments were performed.

2.6. Determination of HSA content

The concentration of HSA was determined with a UV spectrophotometer (Unicam Heλios Alpha, Spectronic Unicam, UK) at 562 nm. The Micro BCA™ Protein Assay (Pierce, Rockford IL, USA) was applied for the determination. The Micro BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a blue colored complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. In the second step of the color development reaction, BCA, a highly sensitive and selective colorimetric detection reagents reacts with the cuprous cation (Cu^{1+}) that was formed in step 1. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations [27].

The test fluid was phosphate buffer, pH 7.2. The concentrations of active agent in the different groups were determined. The calculated value was 13.04% (15 g/(15 g + 100 g)).

3. Results

3.1. Effect on yield

After the preparation of the intermediates, the yields were calculated. It can be seen that the yields of the samples containing HSA were very good (Table 2). The value for S0 was the lowest. The long treatment of this sample could cause a higher ratio of lost particles through the filter system. This was not detected for the other samples, which can be explained by the increase in their particle size, so that the possibility of loss was lower.

3.2. Effect on shape

It can be seen from the SEM pictures that the surface of the original Arbocel A 300 was irregular (Fig. 1). The sticking of the treated particles is clearly visible, with the irregular surface of the cellulose with binder layers of HSA forming bridges between the particles (Fig. 2). A covering layer (''film-like'') of HSA was detected at higher magnification. Numerous HSA spheres 1–2 μm in diameter were also produced during the process (Figs. 3 and 4). This

Table 2
Yield of preparation

Sample	Yield (%)
S0	73.04
S1	96.35
S2	94.26
S3	97.3
S4	94.52
S5	95.39

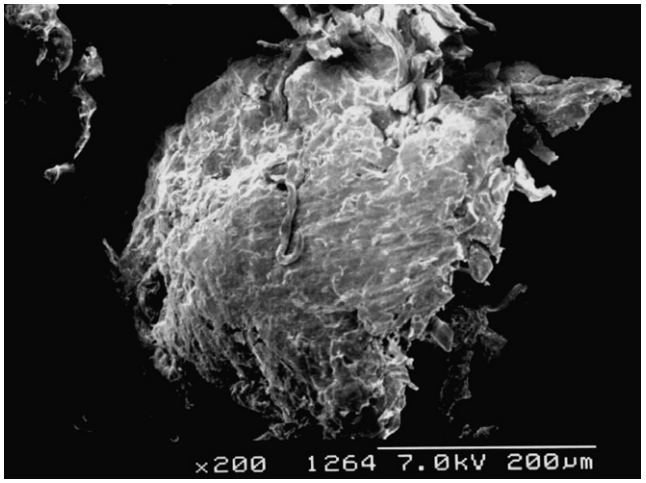

Fig. 1. Arbocel A 300 (SEM 200×).

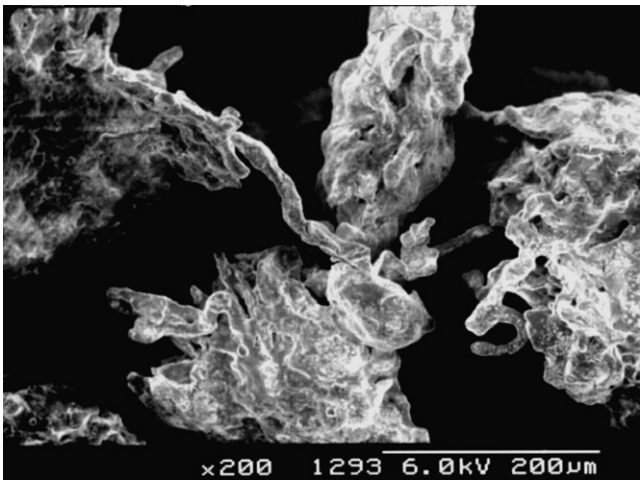

Fig. 2. S4 particle <315 μm (SEM 200×).

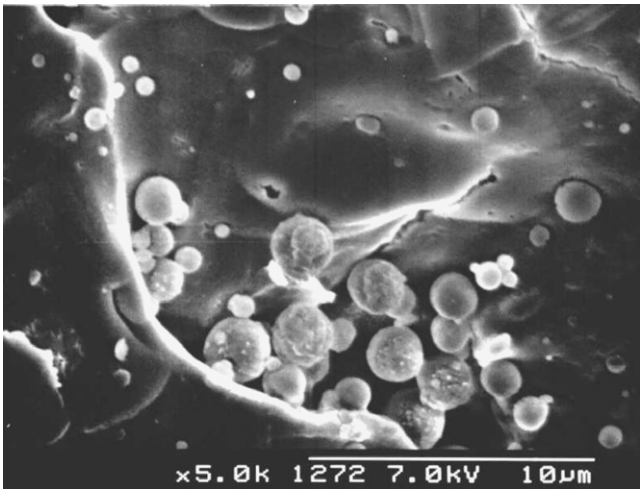

Fig. 3. Surface of S4 (SEM 5000×).

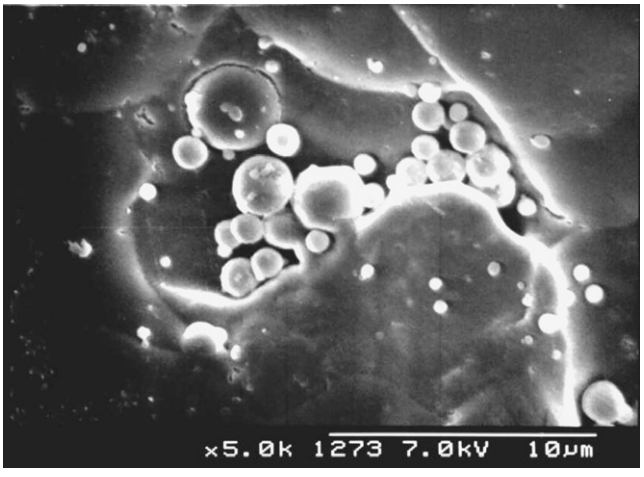

Fig. 4. Surface of S4 (SEM 5000×).

was a consequence of the spray-drying of the solution. These microparticles settled mainly in the irregularities in the powdered cellulose. Because of the size of these particles (smaller than the filter), the loss during the process can be relevant. The covering of the cellulose was very smooth, but a few defects could be detected. These can be due to the incorporation of the spray-dried particles into the layer, and the rupture of the HSA layer and the grained surface (because of the recrystallization of salts) (Figs. 5 and 6).

3.3. Effect on particle size

Because of the previous results, the particle sizes were also determined. It can be seen that the application of HSA caused an increase in the size of the particles (Table 3 and Fig. 7). These results emphasized that HSA was a binder, since the coating could not cause an increase of more than 50% in the diameter. There was no obvious relationship between the concentration of the liquid and the particle size. This can be a result of the complexity of the process. It is known that the viscosity of HSA solution is changing with the concentration [28]. At the lowest concen-

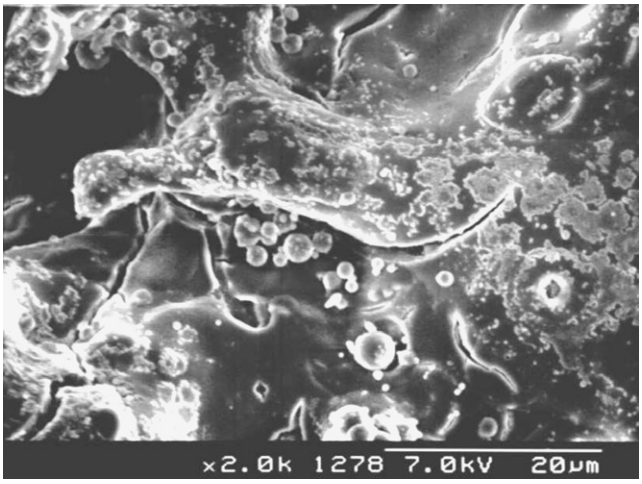

Fig. 5. Surface of S4 (SEM 2000×).

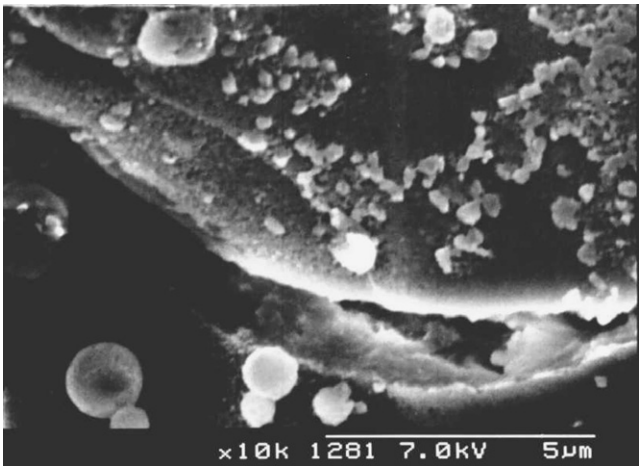

Fig. 6. Surface of S4 (SEM 10000×).

Table 3
Properties of samples

Sample	D50 (μm)	<200 μm	200–315 μm	>315 μm	Flow time (s)
Arbocel A 300	277	10.3	67.9	21.8	13.4 ± 1.6
S0	258	21.3	67.0	12.7	7.7 ± 0.2
S1	466	5.0	10.8	84.3	105.5 ± 26.0
S2	452	4.1	13.6	82.5	46.8 ± 7.9
S3	467	1.9	10.7	87.4	73.4 ± 10.9
S4	406	3.7	23.4	72.9	26.5 ± 5.5
S5	465	1.7	12.4	85.9	231.3 ± 29.1

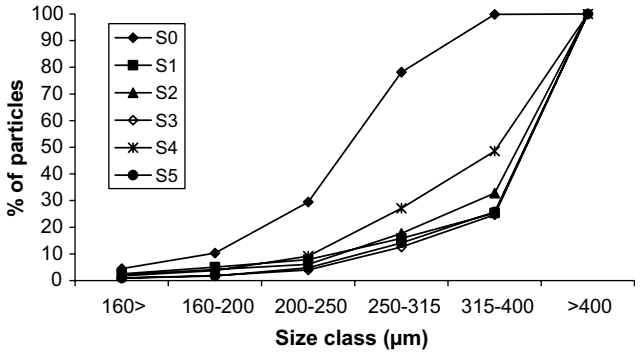

Fig. 7. Cumulative granulometric curve.

tration, the spreading of the liquid on the solid surface is easier (because of the lower viscosity and density), so the covering and the sticking between particles were also higher. In this case however, the processing time was longer and therefore the mechanical stress for these samples was higher, which could cause the abrasion of the products. Hence, an optimum concentration was necessary, where the increase in the aggregates was the lowest (the ratio of the two converse processes was the same). The lowest increase in D50 was detected for sample S4. Thus, this concentration was the most appropriate.

The products were classified according to their particle size. During the fluidization process, the spray-drying of the atomized liquid is inevitable. These particles can be found among the particles smaller than 200 μm. The most appropriate particles measured between 200 and 315 μm; the highest proportion of starting materials is to be found in this range. Thus, the agglomeration is lowest for these particles. The aggregated products were larger than 315 μm.

The proportion of the particles with optimum size was nearly 70% for Arbocel A 300. The ratio of these particles was significantly lower for the samples containing HSA. The highest value was detected for S5, which was ~2 times higher than for the other samples.

3.4. Effect on flowability

It is well-known that the flowability of a sample is determined by the morphological parameters [29]. The flow time of the samples was therefore determined before sieving (Table 3). The flow time was long, with a high deviation, for the particles containing HSA. The best result was detected for S4. This product exhibited the lowest particle size increase. Because of the lower ratio of the irregular sticking of the particles, the shape was better for even flow.

3.5. Effect on content uniformity

The study of the concentration of the active agent revealed that the concentration of HSA was higher in the group containing smaller particles (higher than the calculated value) (Table 4). This can be explained by the spray-drying of the active agent and the abrasion of these spheres from the larger particles (Figs. 3 and 4). The lowest concentration was measured for S3, and in this case the proportion of these particles was also the lowest (Table 4). The formation of these particles was therefore the smallest in this case. Spray-drying is easier for liquids containing a high amount of solid, so the ratio of these particles should be enhanced on increase of the concentration of HSA. However, the larger density and hence the larger viscosity caused a decrease in the sprayability of the liquid. Larger droplets were formed, and thus the evaporation of the liquid took longer. In view of the adverse effects, the optimum value for the concentration of HSA must be found. In this case, 12.5% HSA was optimum. The sticking and the abrasion of the particles were explained previously.

The value for the largest particles could not be exactly evaluated. The determined value was lower than in the other two groups, but it was very variable for the same sample (8.75% was measured for S2, but repetition of this measurement gave >13%, with a high standard deviation (RSD >15%), i.e., the result of the repeated measurements was not comparable). This was influenced by the sampling from agglomerates containing particles with very different particle size.

Table 4
Concentration of HSA (%) in different intermediates

Sample	<200 μm	200–315 μm
S1	16.37 ± 0.94	13.31 ± 0.61
S2	19.10 ± 0.88	12.63 ± 1.25
S3	13.96 ± 0.45	13.49 ± 0.54
S4	18.68 ± 0.83	10.95 ± 1.36
S5	18.90 ± 0.46	14.43 ± 0.28

In the fraction of particles in the interval 200–315 μm, the lowest value was measured for S4 (with the highest RSD). The HSA concentration of the small particles was also high. Accordingly, an uneven distribution of the active agent can be expected. The presence of the active agent could not be evaluated (loss of small particles, or enrichment in the larger particles as a thicker film), because the exact determination of concentration of largest particles was impossible. In spite of its good flowability, S4 was not the best composition because of the uneven distribution of HSA.

4. Discussion

The main aim was to prepare intermediates for the preparation of tablets with different concentrations of HSA. It can be concluded that the yield of the fluidization technique was very good, independently of the concentration of the applied liquid. The process caused an increase in the particle size. The HSA was responsible for the increase, because water did not cause aggregation. The majority of the inert carrier particles were in the range 200–315 μm. During the preparation, the ratio of these particles decreased. The largest proportion was detected for the samples prepared with liquid containing 15% HSA (about 2 times higher than for the second best). In this case, the ratio of the particle size-increasing and destructive processes was the most preferable.

Not only the size, but also the shape of the particles changed, leading to a change in flowability. The concentration of HSA in the fraction containing smaller particles was higher because of the abrasion of the particles and the spray-drying of the HSA. The formation of large aggregates must be eliminated, because the distribution of the active agent is very inhomogeneous.

Finally, it may be stated that this modified layering of powder cellulose with a fluid bed technique can be applied for the production of an intermediate from HSA for the preparation of solid dosage forms. Previously, the appropriate concentration of this protein solution must be optimized, as HSA can act as a binder. Our results indicated that the best value can be reached with liquid containing between 12.5% (most homogeneous distribution of HSA) and 15% HSA (best flowability).

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Research paper

Evaluation of the binding effect of human serum albumin on the properties of granules

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ABSTRACT

The main objective of this study was the application of a solution of human serum albumin as a granulating fluid. The properties of the granules formed were evaluated and compared with those when a conventional binder was applied in the same concentration. The powder mixture contained a soluble (mannitol) and an insoluble component (different types of cellulose). The protein solution applied exerted an appropriate aggregating effect if the system contained microcrystalline celluloses. Powdered cellulose was not suitable for the granulation with human serum albumin solution. As compared with the same concentration of the conventionally applied cellulose ethers as binder, the prepared granules exhibited a larger particle size, a significantly better compressibility, a higher breaking hardness and a favourable deformation process. These findings mainly reflect the good adhesive properties of the protein. The best compressibility and mechanical behaviour were attained on the application of the microcrystalline cellulose Vivapur type 105. This favourable behaviour may be connected with the wettability of cellulose. These results suggest that the formulation of tablets may be easier from an active agent in the serum that binds to albumin (e.g. interferon) since the amount of additives (binder) can be reduced.

1. Introduction

Biologically active peptides and proteins are increasingly becoming a very important class of therapeutic agents because of their extremely specific activity and high tolerability by the human organism [1]. This may afford a possibility for the direct application of these materials. Proteins have a complex internal structure which helps define their biological activity. Any disruption in the primary (amino acid sequence), secondary (two-dimensional structure), tertiary (folding) or quaternary structure (combination of peptide subunits) can result in the deactivation of a protein. Such disruptions may be caused by even the slightest changes in the environment (or even microenvironment) of the protein. The most likely variables which can affect protein structure and stability are related to the temperature, pH, solvent, other solutes and crystallinity states of the protein [2]. These problems must be considered during the formulation of dosage forms containing proteins.

Since proteins may be very sensitive, the main route of administration is by injection. The injection dosage form, however, has a number of disadvantages, such as low patient compliance, and the possibility of infection and pain during repeated injections [3]. This mode of application can be very inconvenient, and the evaluation of non-invasive routes is therefore very relevant.

Various strategies have been pursued to develop safe and effective oral delivery systems for proteins [4] and to develop sustained and long-acting release delivery systems [5,6].

One possibility is the formulation of buccal preparations. For many drugs, and especially peptides and proteins, the buccal route offers many advantages over conventional modes of delivery, with an improved bioavailability due to the avoidance of degradation in the gastrointestinal tract and hepatic first-pass metabolism [7]. Excellent accessibility, high patient acceptance and compliance, and robustness may be mentioned as the attractive features of buccal administration [8].

The formulation of buccal tablets from biological samples is therefore a reasonable demand. Since the applied proteins are mainly to be found in liquids (e.g. serum), their incorporation into tablets can be complex (e.g. first lyophilization step). This process can cause the degradation of the proteins [9]. To protect a protein from freezing (cryoprotection) and/or dehydration (lyoprotection), a protein stabilizer(s) may be used. On the other hand, overuse of an excipient(s) may eventually destabilize a protein.

The simplest means of tablet making is direct compression [10,11]. In this case, many important parameters (e.g. good flowability and compressibility) must be considered [12]. A problem with direct compression is the relatively high compression force, and hence the higher temperature during the compression (formation of "hot spots" [13–15]).

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The preparation of appropriate intermediates can promote the preparation of tablets. The most widely used method is granulation, in which case separation of the proteins from the liquid is not necessary: may serve as a granulating liquid. It is known from our previous results on human serum albumin (HSA) that it forms a film-like layer, and has an aggregating effect, and there is no degradation during the process at 35–40 °C [16]. Evaluation of the binding effect of the protein is therefore reasonable before choice of the appropriate composition. If the binding effect of the protein solution is acceptable, the amounts of other excipients can be reduced, which is very favourable because of the sensitivity of proteins.

In this study, the granulating effect of HSA solution was evaluated and compared with those of various conventionally used binding cellulose ethers. The influence on different powder mixtures was studied. Mannitol (M: as a conventionally used filler in buccal preparations) and different types of cellulose were applied. A high-shear granulator was used to prepare the samples. The possibilities for binding include the film-forming materials applied in the granulating liquid and also the bridges formed from the soluble M after the recrystallization. The parameters of the granules which are of importance as concerns tablet making were tested.

HSA is a single-chain protein synthesized and secreted from the liver cells. HSA (MW 66,472 Da) is found mainly (~50%) in the plasma, where it maintains the pH and osmotic pressure and plays a major role in transporting a wide range of materials such as metal ions, fatty acids, amino acids, metabolites and many drugs (e.g. interferon) [17–20]. HSA is known to be sensitive to heat, ions, etc. [21–23]. The choice of an appropriate formulation (additives and methods) is therefore very difficult.

2. Experimental

2.1. Materials

Mannitol (M) (Ph.Eur. Hungaropharma Plc., Hungary), different types of microcrystalline cellulose (MCC) (Vivapur 101, 103 and 105, J. Rettenmaier & Söhne GmbH & Co. KG, Germany) and powdered cellulose (PC) (Arbocel P 290, J. Rettenmaier & Söhne GmbH & Co. KG, Germany) were applied in powder mixtures.

The granulating liquids were 4% HSA solution (Trigon Biotechnological Ltd., Hungary), an aqueous solution of hydroxypropylmethylcellulose (HPMC, Pharmacoat 606, Shin-Etsu Chemical Co., Ltd., Japan) and hydroxypropyl-cellulose (HPC, Klucel LF, Hercules Inc., USA) in the same concentration.

Parameters of HSA solution (containing also NaCl, KCl, Na_2HPO_4, KH_2PO_4, etc.) were as follows:

- Content of albumin: min. 98% of the total protein.
- Content of endotoxin: 5 IE/mg.
- Content of chloride: 4.85–5.35 mg/ml.
- Osmolarity: 285.0–315.0 mosmol/kg.

2.2. Preparation of samples

The samples were prepared in a high-shear granulator (ProCepT 4M8 granulator, ProCepT nv, Belgium).

The powder mixture was prepared from 100 g M and 100 g cellulose. The type of cellulose and the composition of the granulating liquid were varied (Table 1). The amount of water was the same for all the samples. The constant operational parameters were determined in the previous experiments:

- Impeller speed: 750 rpm.
- Chopper speed: 3000 rpm.
- Dosing speed: 5 ml/min.

Table 1
Compositions of samples

Sample	Cellulose	Granulating liquid	Amount of liquid (g)
S1	MCC 101	4% HSA	80
S2	MCC 101	Water	76.8
S3	MCC 101	4% HPMC	80
S4	MCC 101	4% HPC	80
S5	MCC 103	4% HSA	80
S6	MCC 103	Water	76.8
S7	MCC 105	4% HSA	80
S8	MCC 105	Water	76.8
S9	P290	4% HSA	80
S10	P290	Water	76.8

- Spheronization time: 1 min.
- Total granulation time: 17 min.
- Drying: on trays at 40 °C for 2 h.

2.3. Evaluation of samples

The sizes and the size distributions of the samples were evaluated with an analytical sieve (Retsch GmbH, Germany) and a sieving system software (Retsch EasySieve 2.0, Germany). Particles larger than 2 mm were regarded as waste. The yield calculations and all tests were performed after the removal of these particles.

A powder testing apparatus (PTG-1, Pharma Test GmbH, Germany) was used to test the time of the flow of 100 ml of sample. A teflon accessory with an orifice 10 mm in diameter was applied.

The surface tensions of the HSA, HPMC and HPC solutions and the water were measured with a ring method (Krüss GmbH, Germany). A Brookfield LVDV-II viscosimeter with CPE 42 spindle (Brookfield Engineering Laboratories Inc., USA) was used for the determination of the viscosity of the solutions at 25 °C. One milliliter sample was tested at 12 rpm.

Densities (bulk (ρ_0) and tapped (ρ_∞)) were determined with a STAV 2003 Stampfvolumeter (Engelsmann A.G.L., Germany). Carr's index was calculated from these results [24] and three parallel tests were carried out:

$$\text{Carr's index} = \frac{\rho_\infty - \rho_0}{\rho_\infty} \times 100$$

The breaking hardness was tested for granules measuring between 710 and 800 μm. This device contains a special specimen holder and a stamp, and is connected to a computer via an interface; thus, not only can the ultimate deformation force be measured, but the process (force–time and force–displacement curves) can also be followed. If the measured plot (force–time) is parallel to the *x*-axis the deformation is viscoelastic; if the plot

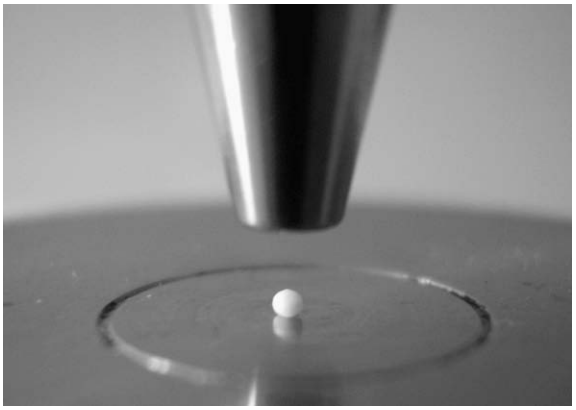

Fig. 1. Sample holder of breaking hardness tester.

rises linearly, the deformation is elastic. The specimen is located horizontally and the stamp moves vertically (Fig. 1). Twenty parallel measurements were performed.

The measuring range was 0–200 N, the speed of the stamp was 20 mm/min, the output was 0–5 V, and the sensitivity was ±0.5% ± 0.1 digit. The sensor was UNICELL force measuring equipment, calibrated with the C9B 20 kN cell.

The Enslin number is a simple semi-quantitative measure of the water uptake of a powder, and is equal to the amount of fluid absorbed by 1 g of the powder (ml/g). An Enslin apparatus with a glass filter and a pipette with 0.01 ml accuracy were used for these experiments. A monolayer of particles took up the maximum quantity of water possible through a filter paper under these conditions. Each powder (0.5 g) was tested; 5 parallel experiments were performed.

Statistica for Windows 7.1 AGA software (StatSoft, Inc. Tulsa, USA) was used for the statistical analysis. The two-sample *T-test* was applied for the comparison of two groups of results. The confidence interval was 95% ($p < 0.05$).

3. Results and discussion

3.1. Particle size

The yields of the samples containing MCC were very good (Table 2). The values for powdered cellulose (S9 and S10) were the lowest because large, very loose flakes were formed in these cases.

The particles of samples produced with the well-known binder were smaller than those of S1. The particles of the samples containing HSA were larger than those of the samples containing the same powder mixture, but prepared only with water (S2, S6, S8 and S10).

The conventionally used binder in the same concentration exhibited lower particle sizes than that of the sample prepared with HSA. The explanation may be the different wetting of the powder mixture and the different binding potency of the binder. The surface tensions of the liquids, which can influence the wetting of the powder mixes, were 42.66 ± 0.62 Nm for HSA solution, 44.92 ± 0.94 Nm for HPMC solution and 40.26 ± 0.43 Nm for HPC solution. There was no relevant difference in this parameter, which was therefore not the main factor responsible for the particle size of the granules. The spreading of the liquid can be different because of variant viscosity (1.5 mPas for HSA solution, 19.1 mPas for HPMC solution and 45.9 mPas for HPC solution). The particle size was lower for granules prepared with viscous solution. The favourable granulating capacity of HSA solution can probably rather be explained by the better binding capacity of HSA solution.

For the evaluation of the effects of the starting materials, different parameters of the MCCs and liquids were considered. The particle sizes, surface tensions, bulk densities and Enslin numbers for water uptake of the different MCCs were determined.

MCCs with higher bulk densities exhibited higher Enslin numbers (Table 3). A higher amount of fibres can bind a higher amount of liquid. When the water uptake was higher, the D50 value for the sample prepared with HSA solution was also higher (Table 2; S1, S5, S7 and S9). The corresponding tendency was opposite for the samples produced with water. The explanation may be that the higher amount of water taken up by MCC cannot act as a binder, since this material is not soluble. The only binder in this composition is the recrystallized M. The proportion of the water available as the solvent of M was therefore lower, and accordingly these granules were smaller. The liquid taken up by the MCC also contained HSA, for the samples were prepared with HSA solution. After drying, this component formed bridges between the particles, and in this case the most important binder was not the recrystallized M, but the HSA. When the amount of granulating fluid taken up was higher, there was a higher possibility of formation of more HSA bridges, and hence the mean size of the samples was higher.

Table 2
Granules size distribution

	Yield (%)	D10 (mm)	D50 (mm)	D90 (mm)
S1	99.21	0.607	0.885	1.284
S2	91.85	0.111	0.608	1.195
S3	87.16	0.114	0.687	1.139
S4	87.06	0.188	0.301	0.904
S5	90.70	0.122	1.054	1.411
S6	84.95	0.068	0.334	0.743
S7	85.53	0.437	0.837	1.194
S8	90.60	0.287	0.696	1.130
S9	57.09	0.248	0.628	1.288
S10	57.45	0.150	0.574	1.272

3.2. Flowability

The properties of the granules were determined. Their flowability and compressibility were better than those of the starting powder mixtures (Table 4). The flow times for the samples with water (S2, S6, S8 and S10) were slightly lower than those for the samples containing binder. This difference was not of importance since these values are very good. The bulk densities of the samples prepared with HSA (S1, S5, S7 and S9) were higher than those of the granules prepared with water (except for S7 and S8, where there was no significant ($p < 0.05$) difference). This parameter was considerably lower for the samples prepared from powdered cellulose (S9 and S10) because they formed large fluffy flakes. The compressibility (Carr index) is another important parameter in tablet making. It was significantly better for the samples containing different binders. This can be explained by the better distribution of the particle size. HSA at this concentration caused a more appreciable increase than the conventional binders.

Table 3
Properties of MCC

Type	Particle size* (μm)	Bulk density* (g/cm^3)	Enslin number (ml/g)
101	50	0.29	2.91 ± 0.05
103	50	0.32	3.03 ± 0.06
105	25	0.23	2.45 ± 0.12

* Data from producer.

Table 4
Properties of granules and powders

Sample	Flow time (s)	Bulk density (g/cm^3)	Carr index (%)	Breaking force (N)
S1	8.0 ± 0.12	0.706 ± 0.011	4.87 ± 0.44	2.64 ± 0.29
S2	7.1 ± 0.15*	0.639 ± 0.006*	11.15 ± 1.39*	2.43 ± 0.55
S3	7.7 ± 0.12	0.670 ± 0.003*	7.69 ± 0.77*	2.44 ± 0.39
S4	6.3 ± 0.12*	0.624 ± 0.001*	7.44 ± 0.44*	1.93 ± 0.36*
S5	8.8 ± 0.6	0.658 ± 0.002	11.79 ± 0.59	2.11 ± 0.35
S6	6.5 ± 0.12**	0.568 ± 0.010**	14.10 ± 0.44**	1.97 ± 0.38
S7	7.9 ± 0.15	0.714 ± 0.007	4.62 ± 0.77	2.70 ± 0.63
S8	7.4 ± 0.12**	0.723 ± 0.006	8.53 ± 0.62**	2.49 ± 0.35
S9	10.2 ± 0.21	0.258 ± 0.005	6.67 ± 0.44	2.36 ± 0.41
S10	9.8 ± 0.25	0.272 ± 0.005**	9.23 ± 3.35	2.28 ± 0.5
MCC101 + M	34.2 ± 6.38	0.399 ± 0.006	24.87 ± 1.94	–
MCC103 + M	31.9 ± 2.2	0.434 ± 0.0004	23.98 ± 2.83	–
MCC105 + M	No flow	0.455 ± 0.012	28.66 ± 2.16	–
PC + M	19.8 ± 0.93	0.434 ± 0.010	25.90 ± 1.60	–

* Significant difference between the sample and S1.
** Significant difference between the samples prepared from the same powder mixture, but with different granulating fluid.

3.3. Mechanical property

The mechanical properties of the granules were better for S1 than for the compositions containing the conventional binders. Higher values were found for the samples containing HAS (S1, S5, S7 and S9), independently of the type of cellulose. The best mechanical properties were those of the sample containing MCC 105 (S7 and S8). The water uptake of this sample was lowest because of the low density. The number of fibres and thus the number of bridges formed may be lower, but the binding may be stronger and accordingly the hardness may be higher. The higher possibility of the binding force of recrystallized M also must be considered. The texture of this sample was therefore the most compacted.

Not only the breaking hardness, but also the deformation process can provide information on the processibility. The breaking curve of S1 (Fig. 2) was very similar to those of the compacted pellets or granules [25]. There were three phases: a short elastic part was followed by a viscoelastic phase, and finally an elastic section up to the breaking point. There were no meaningful irregularities in the curves, which revealed only small deformations caused by the slightly inhomogeneous structure (Fig. 3). S2, prepared with water, exhibited a primarily elastic curve with a short viscoelastic section (Fig. 4). It is well known that air exhibits elastic properties,

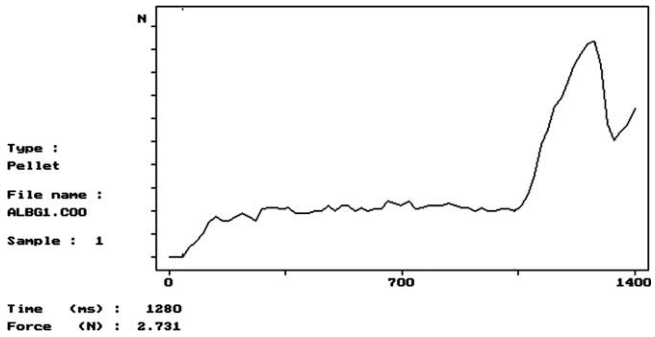

Fig. 2. Breaking hardness curve of sample S1 (granulating fluid: HSA solution).

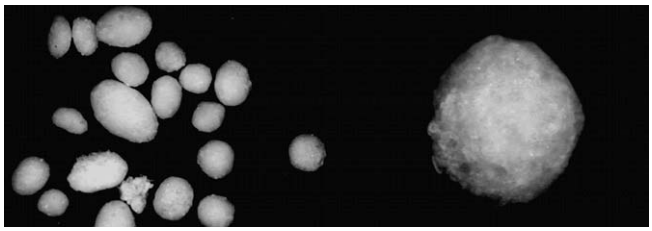

Fig. 3. S1 particles (magnification: 10× (left) and 50× (right)).

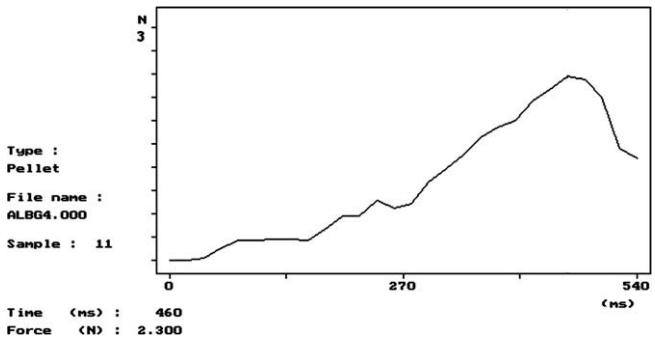

Fig. 4. Breaking hardness curve of sample S2 (granulating fluid: water).

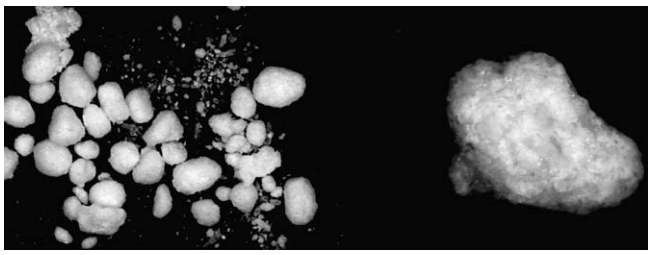

Fig. 5. S2 particles (magnification: 10× (left) and 50× (right)).

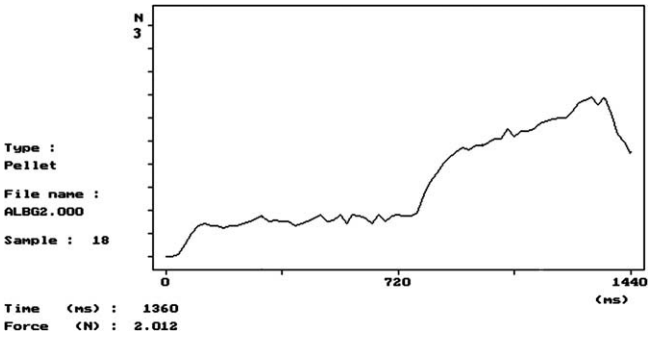

Fig. 6. Breaking hardness curve of sample S3 (granulating fluid: HPMC solution).

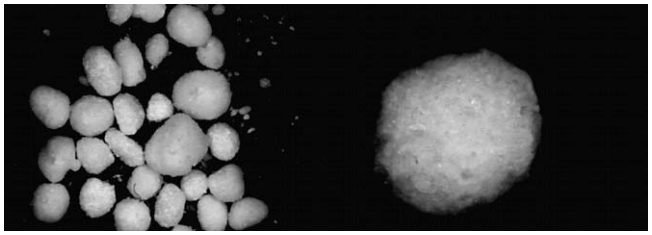

Fig. 7. S3 particles (magnification: 10× (left) and 50× (right)).

and in this case the amount of entrapped air was higher because of the loose structure and irregular shape (Fig. 5). More elastic materials cannot be compressed because of the capping [10]. The curve of S3 (Fig. 6) was better than that of S2, but there were more irregularities than for S1, and the separation of the different phases was also less marked. The shape of these particles was very similar to that of S1 particles (Fig. 7).

4. Conclusions

It can be concluded that the HSA solution had a very good granulating effect when the system contained the studied MCCs. Powdered cellulose was not appropriate for granulation with HSA solution. As compared with the conventionally used binder in the same concentration, the granules formed with HSA displayed a larger particle size, a significantly better compressibility, a higher breaking hardness and a favorable deformation process. The explanation of the advantageous properties is the good adhesive properties of the protein. The different MCCs furnished products with different properties. This was connected with the structure of the particles and their water uptake. According to our results the best compressibility (highest bulk density), and the best mechanical behaviour were detected for materials containing MCC 105 and granulated with HSA solution.

It may be stated that the inclusion of HSA in the granulating fluid can be very useful: pretreatment of this component is not

necessary, the granulating effect is considerable and the amounts of additives can be decreased.

Acknowledgement

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III.

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RESEARCH ARTICLE

Effect of lubricant on spreading of coating liquid on surface of tablets containing pancreatin

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Abstract

The objective of this study was to evaluate the spreading of the coating liquid on different tablets containing pancreatin and microcrystalline cellulose. The effects of the ratio of the components, the presence of magnesium stearate and the blending circumstances were investigated. The contact angle of the liquids on the different tablets did not change linearly. For the mixture containing 50% pancreatin, the deviation of the measured value from the predicted one was more than 25%. This deterioration was also detected for mixtures containing 1% lubricant, but the extent was lower and was not modified by change of the mixing circumstances. This phenomenon was explained by the special microstructure of the surface of the tablet. This was predicted from the spreading coefficient, calculated from the surface free energy. The enrichment of pancreatin on the surface was preferred in binary mixtures. The spreading of magnesium stearate was most preferred for the powder mixture, and thus prediction of the properties of the tablet was easier for these mixtures. The extent of the effect of this excipient on the surface properties was very wide-ranging. The change in the spreading of the coating liquid was significant; however, the change in the work of friction was negligible.

Keywords: *Coating; contact angle; magnesium stearate; spreading coefficient; surface free energy*

Introduction

Pancreatin (P) is a combination of digestive enzymes that is secreted by the pancreas. It is prepared from the pancreas of pig or ox and consists of lipases, amylases and proteases; it is therefore able to break down fats, starch and proteins. Its preparations are taken orally with, or just before food. It is processed to the most appropriate form by the pharmaceutical industry for the treatment of given pathophysiological problems.[1] It is administered in cases of chronic pancreatic insufficiency to restore normal digestive conditions in the intestines,[2] and for the treatment of cystic fibrosis,[3] and it is also used as an additive in the food industry.[4,5]

The components of P are proteins.[6–8] They have complex internal structures which determine their biological activity. Any disruption in the primary (amino acid sequence), secondary (two-dimensional structure), tertiary (folding) or quaternary structure (combination of peptide subunits) can result in the deactivation of a protein. Such disruptions may be caused by even the slightest changes in the environment or even the microenvironment of the protein. The most likely variables which can affect protein structure and stability are related to temperature, pH, solvent, other solutes and the crystallinity state of the protein.[9,10] These problems, among others, must be considered during the formulation of dosage forms containing proteins.

P is mainly incorporated into solid dosage forms.[11] Wet or melt granulation is not appropriate for the preparation of granules containing this protein because the pancreatic enzymes can be damaged by the simultaneous effects of moisture and heat.[12] The preferred way for the formulation of a solid dosage

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form is direct compression.[13,14] In this case, not only the conventional parameters such as the properties of the surface, homogeneity, good flowability and good compressibility of the powder mixture[15] must be considered, but also the thermal stress generated during the high-speed compression performed under high pressure.[16,18–19] The destructive effect of the gastric juice may also be very important.[12,17] P exerts its action in the intestines, and the coating of solid dosage forms with an enteric resistant polymer is therefore a step very often used in this case.[12] The parameters of this process can influence the efficacy of this enzyme.

Many pharmaceutical processes involve interactions at interfaces. Interactions between a liquid and a solid are particularly common.[20] A successful film coating requires the spread of a liquid over a tablet surface.[14] The contact angle is used to describe the situation. If a drop of liquid is placed on a flat, smooth, horizontal solid surface, it may spread completely, but it is more likely to form a drop. This drop will exhibit a definite angle relative to the solid, known as the contact angle. The contact angle depends on the compounds involved and the spreading of the components on the surface.[21] Various properties of the liquid, such as its surface tension, polarity, viscosity, etc., can influence this process.[22] The surface of the tablets can also change their wetting.[23] The most important parameters are the surface free energy, surface roughness, porosity, etc. It is known that several components in the powder mixture can change the surface properties of the comprimates.[24] An example of this phenomenon is the enrichment of hydrophobic magnesium stearate (Mg-st) on the surface of tablets.[25] The mixing of this component is generally critical. A short blending is necessary at the end of the mixing process. The effects of Mg-st on the microstructure of the tablet surface and hence on the subsequent technological step were evaluated previously, but not all aspects of the coating were fully investigated.

Our present aim was to study the spreading of a coating liquid on the surfaces of different tablets containing P and different amounts of microcrystalline cellulose (MCC) and Mg-st as excipients, prepared by direct compression. The contact angles of the aqueous dispersion forming an enteric soluble coat on the tablets were determined. The microstructure of the tablet surface was predicted from the spreading coefficient, which was calculated from the surface free energy. The effects of the mixing method and the mixing time on the wettability of the tablets were also tested. The preformulation compactibility test is very important, so the effects of the components in various compositions were examined with an instrumented tablet machine and the results were compared with those of wettability studies.

Materials and methods

Materials

P (Ph.Eur. Richter Gedeon Nyrt., Budapest, Hungary) (D50 = 134 μm) was applied as active agent. The excipents used for the tablet manufacture were MCC (Avicel PH 101, FMC Europe N.V., Brussels, Belgium) and Mg-st (Baerlocher GmbH, Unterschleissheim, Germany). A 20% aqueous dispersion of Acryl EZE (methacrylic acid-ethyl acrylate copolymer [1:1]) (Colorcon Ltd., Dartford, Kent, UK) was used as coating liquid.

Preparation of powder mixtures

The first group of powder mixtures contained only P and MCC different ratios. The contents of P were 0, 10, 20, 30, 40, 50, 60, 80 and 100%. The second group of powder mixtures contained the same ratios of P and MCC, but also 1% Mg-st. This concentration of Mg-st is conventionally used in tablet making.[14] In both cases, the P and MCC were mixed in a Turbula mixer for 5 min at 50 rpm. The lubricant was added to this mixture and mixing was continued for an additional 1 min.

Next the effects of the mixing circumstances were tested. The applied powder mixture contained 50% P, 49% MCC and 1% Mg-st. The mixing times were 2, 6 and 11 min. The process was performed in one step (all components were mixed together) and also in two steps (the P and MCC were mixed for 1, 5 or 10 min and the lubricant was then added, with mixing for an additional 1 min).

Preparation of comprimates for measurement of contact angle

Contact angle was measured on flat comprimates of 12 mm in diameter, prepared with a hydraulic press (Röltgen GmbH & Company KG, Sollingen, Germany) at 1 MPa.

Measurement of contact angle

The spreading of 12 μL of Acryl Eze dispersion on the tablet surface was studied via its contact angle. A drop shape analyzer was applied (Krüss DSA 10, Krüss GmbH, Hamburg, Germany). The diameter of the needle was 0.8 mm (Sterican 0.8 × 2.2 mm 21G × ⅞ inch B.Braun Melsungen AG., Melsungen, Germany). Ten parallel experiments were performed.

Calculation of surface free energy

The microstructure of the tablet surface was predicted from the spreading coefficient, which was calculated

from the surface free energy. An indirect method of assessing the surface free energy (γ) from wettability measurements is widely used.[26,27] In the method of Wu,[28] the surface free energy is taken as the sum of dispersive (γ^d) and polar (γ^p) components. The surface free energy of solid materials can be determined by means of contact angle measurements with two different liquids with known polar and disperse part of surface tension properties. They can be assessed by solving an equation with two unknowns:

$$(1+\cos\Theta)\gamma 1 = \frac{4(\gamma_s^d \gamma_1^d)}{\gamma_s^d + \gamma_1^d} + \frac{4(\gamma_s^p \gamma_1^p)}{\gamma_s^p + \gamma_1^p} \quad (1)$$

where Θ is the contact angle, γ_s is the solid surface free energy and γ_l is the liquid surface tension [superscripts referred to their polar (γ^p) and dispersive part (γ^d)].

If the surface free energy of the solid materials is known, the spreading coefficient (S) may be computed and the interactions between the two materials may be predicted. The spreading coefficient is calculated as the difference between the adhesion work and the cohesion work. The two materials which interact can be two powders, a powder and a liquid, or any material and the equipment. The spreading coefficient (S_{12}) of a material (1) over the surface of another material (2) can be determined as follows:[29]

$$S_{12} = 4\left[\frac{4(\gamma_1^d \gamma_2^d)}{\gamma_1^d + \gamma_2^d} + \frac{4(\gamma_1^p \gamma_2^p)}{\gamma_1^p + \gamma_2^p} - \frac{\gamma_1}{2}\right] \quad (2)$$

An optical contact angle - measuring device (OCA 20, DataPhysics Instruments GmbH, Filderstadt, Germany) was utilized to determine the wetting properties of the samples. The test fluids were distilled water and diiodomethane (Merck KGaA, Darmstadt, Germany). According to Ström,[30] the dispersion part of the surface tension was 21.8 mN/m for water and 50.8 mN/m for diiodomethane. The polar part of the surface tension was 51 mN/m for water and 0 mN/m for diiodomethane. Compacts of 0.50 g of powder mixtures were made with a hydraulic press (Specac Inc, Graseby, UK), with a dwell time of 10 s, at a pressure of 200 MPa. Circle fitting was applied to determine the contact angle formed on comprimates prepared from different samples.

Friction and plasticity studies

The effects of the excipients on the compactibility were also studied. Pure P and powder mixtures containing 50% P with or without Mg-st were compressed into tablets with a Korsch EK0 instrumented eccentric tablet machine (Emil Korsch Maschinenfabrik, Germany). The strain gauges allow the pressure forces on the upper and lower punches to be followed with force-measuring equipment, which was calibrated with a Wazau HM-HN-30 kN-D cell (Kaliber Ltd, Hungary). The displacement transducer was fitted over the upper punch. The transducer distance accuracy was checked by using five measuring pieces of different thicknesses (2.0, 5.0, 7.5, 10.0 and 15.0 mm) under zero load (Mitutoyo, Tokyo, Japan).
The parameters were as follows:

Air temperature: 24–25°C;
Relative humidity of air: 45–50%;
Tablet mass: 300 ± 5 mg;
Speed of compression: 36 tablet/min;
Compressing force: 10 ± 1 kN;
Punch: 10 mm, biconvex;
Number of tablets recorded: 10.

The lubrication coefficient (R) can be calculated from the upper (F_{upper}) and lower (F_{lower}) forces.[31] This coefficient refers to the loss of force due to the internal and external friction:

$$R = F_{lower}/F_{upper} \quad (3)$$

The plasticity was calculated according to Stamm and Mathis ($PL_{S\text{-}M}$):[32]

$$PL_{S-M} = \frac{E_2}{E_2 + E_3} \times 100 \quad (4)$$

The friction work was also determined from the detected values. De Blaey and Polderman[33] defined the work of friction as the integral of the difference between the upper and lower punch forces. Later, Järvinen and Juslin[33] presumed that the movement of the particles varies linearly with the distance of the upper punch. According to the Unckel equation,[35] the distribution of the axial force decreases exponentially from the upper to the lower punch:

$$W_{fric} = \int_{s1}^{s2} \frac{F_U - (F_U - F_L)}{\ln F_U/F_L} ds \quad (5)$$

where the height of the powder column changes from s1 and s2 during the compresion, F_U is the upper force and F_L is the lower punch force.[36]

Statistical tests

Statistica for Windows 7.1 AGA software (StatSoft, Inc. Tulsa, USA) was used for the statistical analysis. The two-sample *t*-test was applied for the comparison of

two groups of results. The confidence interval was 95% ($P < 0.05$).

Results and discussion

Study of contact angle on tablets containing different ratios of pancreatin

It can be seen from the results that the wetting of pure P was not the most appropriate for the coating (Table 1). Pure MCC exhibited significantly lower contact angles. The application of Mg-st (contact angle: 92.7 ± 2.2°) significantly ($P < 0.05$) increased the contact angle. The extent of this alteration was not proportional. The increase was 10.6° for MCC and 5.8° for P.

The change in the contact angle was evaluated in correlation with the concentration of the active agent. The correlation of linear fitting (R^2) was 0.9099 for mixtures without Mg-st, and 0.9344 for those with Mg-st (Figure 1). Thus, it can be concluded that the tendency was not linear; as a linear change was expected, further investigation of the mixtures was necessary. The theoretical values of the contact angle were determined from the results on the pure materials, and the differences between the theoretical and measured values were studied. A linear fitting between the parameters of the starting components was applied for the determination of the theoretical contact angle of the mixtures. This calculation was performed for the samples with and without Mg.-st. The absolute and relative deviations are presented in Table 2. Since the signs of these values were positive, the relevance of the more hydrophobic material can be detected. This phenomenon is undesirable for the coating. In both cases, the maximum difference was detected at 50%. The relative change was more than 25% for the sample prepared without lubricant. The application of Mg-st led to decreased differences from the predicted values.

Study of effects of mixing circumstances

The mixing time and method were tested for the mixture containing 50% P, the composition for which the deviation from the theoretical value was highest. In the first step, the mixtures without Mg-st were prepared using different mixing times. There was no significant effect of this parameter on the wetting of the tablets prepared from these mixtures (Table 3). The blending of the lubricant was performed in one step or in two steps, when the mixture of P and MCC was previously produced and the Mg-st was added to this blend. Significant differences did not occur on variation either of the mixing time or of the method.

It can be seen that the difference between the predicted and measured values was not changed by altering the duration of mixing or the method. It was nearly 10° in each case. The microstructure of the tablet surface did not vary. The arrangement of the components can therefore be explained by an independent phenomenon. In this study, the effect of the surface free energy was investigated. This parameter was calculated from the wettability of the components. The lowest surface free energy was detected for Mg-st, and the highest for MCC (Table 4).

The spreading coefficient was calculated from this data. When the sign of the spreading coefficient (S_{12}) is positive, material 1 spreads on the surface of material 2. Every combination of the components was tested, and the positive cases are listed in Table 5. It can be seen that P covered the MCC particles. Since the wettability of pure P is poorer than for MCC, this arrangement

Table 1. Contact angles on tablets containing different ratios of pancreatin (P) and MCC.

P (%)	Without Mg-st (°)	With Mg-st (°)
0	29.5 ± 1.7	40.1 ± 2.1
10	36.3 ± 1.0	46.5 ± 1.8
20	44.1 ± 1.7	51.5 ± 1.6
30	50.4 ± 1.6	58.4 ± 1.9
40	58.2 ± 1.2	64.5 ± 2.1
50	65.9 ± 1.3	69.5 ± 2.3
60	67.6 ± 1.5	72.9 ± 2.1
80	72.5 ± 1.8	76.2 ± 1.9
100	74.6 ± 1.9	80.4 ± 1.4

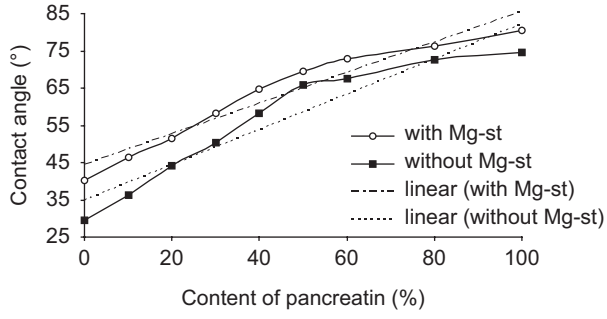

Figure 1. Contact angles for mixtures without magnesium stearate and with magnesium stearate.

Table 2. Deviation of measured contact angle from the theoretical value.

	Without Mg-st		with Mg-st	
P (%)	(°)	(%)	(°)	(%)
0	0	0	0	0
10	2.3	6.7	2.3	5.2
20	5.6	14.5	3.3	6.8
30	7.3	17	6.2	11.8
40	10.6	22.2	8.5	15.1
50	13.8	26.6	9.3	15.3
60	11	19.5	8.7	13.5
80	6.9	10.5	3.9	5.4
100	0	0	0	0

Table 3. The effects of the duration of mixing and the method on the contact angle.

Without Mg-st		With Mg-st			
		One step		Two steps	
Mixing duration (min)	Θ (°)	Mixing duration (min)	Θ (°)	Mixing duration (min)	Θ (°)
1	65.6 ± 1.8	2	69.9 ± 1.8	1 + 1	68.9 ± 1.8
5	65.9 ± 1.3	6	69.1 ± 1.9	5 + 1	69.3 ± 2.2
10	64.9 ± 1.7	11	69.4 ± 1.8	10 + 1	68.1 ± 2.2

Table 4. Wettability and surface free energy of components.

Sample	Θ_{water} (°)	$\Theta_{diiodomethane}$ (°)	γ^d (mN/m)	γ^p (mN/m)	γ^{tot} (mN/m)
P	36.34 ± 1.97	51.76 ± 1.85	30.65	32.84	63.49
MCC	25.98 ± 1.96	15.09 ± 1.92	44.27	32.15	76.42
Mg-st	96.07 ± 1.87	62.38 ± 1.87	26.77	4.37	31.14

Table 5. Spreading coefficient (mN/m).

Material 1	Material 2	S_{12}
P	MCC	10.4
Mg-st	MCC	19.8
Mg-st	P	10.3

Table 6. Compactibility parameters.

	R	PL_{S-M} (%)	FW (Nm)
MCC	0.89 ± 0.003	80.8 ± 0.64	0.296 ± 0.019
P	0.92 ± 0.002	77.9 ± 1.15	0.142 ± 0.015
MCC+P	0.93 ± 0.002	79.1 ± 0.65	0.153 ± 0.011
MCC+P+Mg-st	0.94 ± 0.004	78.2 ± 1.30	0.150 ± 0.016

explained the higher measured contact angle than expected from the theoretical value. Mg-st covered both of the other components. The spreading coefficient was higher for MCC. Since Mg-st covered both components, the difference between the theoretical and measured values was decreased.

Friction and plasticity studies

The R, PL_{S-M} and FW values were calculated for both main components and for mixtures containing 50% P (Table 6). The highest FW and lowest R were detected for MCC, but PL_{S-M} was highest for this component. P exhibited better surface properties (lower FW) and poorer PL_{S-M}. For the binary mixture, PL_{S-M}, which describes the bulk property, was nearly the same as the calculated value (average of the data for the pure materials). The parameters relating to the surface properties were also closer to the values for P. This was in accord with the findings of the wettability study.

The properties of powder mixtures with and without Mg-st exhibited very similar behavior. Slightly better R values were measured for the samples containing Mg-st. A significant effect was detected in the wettability of these powder mixtures, but this phenomenon was not detected in the compactibility parameters.

Conclusions

The extent of spreading of the coating liquid to form an enteric soluble film on the tablets decreased with increasing amount of P. However, the change in this parameter was not proportional to the concentration of this component. The deviation from linearity was highest for the mixture containing 50% P. A deviation was also detected in the presence of 1% Mg-st, but its extent was then lower. The mixing circumstances did not cause significant change in this parameter. The enrichment of the P on the surface of tablets prepared from binary mixtures was supported by the spreading coefficient between the components. The spreading coefficient of Mg-st revealed that both components were covered by this lubricant. The deviation from the predicted wettability was therefore decreased when this excipient was applied.

The plasticity was not affected in a similar way as the wettability, for it is a bulk property. The alteration in the friction exhibited a similar tendency to the spreading of the coating liquid on tablets containing powder mixtures. Thus, a change in composition caused more appreciable alterations in the properties of the surface of the tablets than in the tablettability. Mg-st caused less change in the friction than in the wettability.

The determination of surface free energy can be a useful tool for prediction of the microstructure of the surface of tablets. The lubricant-induced changes in the properties of the tablet can differ for the different properties. These parameters must be investigated, because these effects can be very important in the formulation of coated dosage forms.

Declaration of interest

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IV.

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Optimization of the formulation of solid multiparticulate dosage forms containing pancreatin

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ABSTRACT

The objective of this study was to investigate the changes in the starch-hydrolysing activity of pancreatin under conditions that can occur during the formulation of multiparticulate systems (granulation, direct compression for the preparation of minitablets and coating). Direct compression did not induce a significant alteration in the starch-hydrolysing activity. A factorial design was applied in the testing of the wet conditions ensured by water and ethanol. These had a significant impact, though ethanol caused a more relevant decrease. An increased content of liquid was necessary for unwanted effects, but the changes of its amount in the tested range were not highly relevant. In both cases, the most important factor in the investigation of wet conditions was temperature. During the study of the effects of modelling of the circumstances of tabletting, elevated temperature did not cause alterations in the relatively dry material. This information can promote an improved design for the preparation of the dosage form. Pelletization is not appropriate for the preparation of an intermediate. Direct compression is the most suitable formulation step. Coating must be performed at low temperature with aqueous systems, but rapid drying is also advisable for the first separating layer. A mathematically based optimization can be necessary for the preformulation study of the preparation of dosage forms containing sensitive enzymes.

Keywords: Direct compression; Factorial design; Pancreatin; Starch-hydrolysing activity; Wet conditions

1. Introduction

Pancreatin (PAN) is a combination of digestive enzymes secreted by the pancreas. It is prepared from the pancreas of pig or ox and consists of lipases, amylases and proteases; it is therefore able to break down fats, starch and proteins. It is administered in cases of a chronic pancreatic insufficiency to restore normal digestive conditions in the intestines (Parfitt, 1999) and for the treatment of cystic fibrosis (Patchell et al., 2002), and it is also used as an additive in the food industry (Kong et al., 2007).

The components of PAN are proteins (Reetz, 2002; Hill et al., 2008; Tripathi et al., 2008). These have complex internal structures which determine their biological activities. Therapeutically applied PAN is mainly incorporated in coated multiparticulate solid dosage forms. Popular multiparticulate systems are minitablets or granules/pellets filled into capsules (van der Merwe et al., 2004). Different methods are available for the formulation of tablets (Ritschel and Bauer-Brandl, 2002; Aulton, 2007), while a widely used possibility for the preparation of minitablets is direct compression. For this active agent, it is necessary to consider not only the conventional parameters, such as the properties of the surface, homogeneity, good flowability and good compressibility of the powder mixture (Rubinstein, 1987), but also the high pressure on the special internal structure of the active component and the thermal stress generated during high-speed compression performed under high pressure. It is well known that the generation of heat is unavoidable during the preparation of tablets (Lieberman et al., 1989; Rankell and Higuchi, 1968). The heat generated is high for tablets prepared by direct compression, since a high compression force must be applied. It is also well known that if crystals are arranged side to side with a high thermal conductivity edge, then this promotes the attainment of a higher temperature in a very small volume. This increased temperature can be higher than the melting point of the material and the crystals will melt (nearly 100 °C can be reached). Since melted materials recrystallize after com-

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pression, the particles lose their individuality. Such sites in the texture are called "hot spots" (Kedvessy and Garamvölgyi-Horvát, 1973; Fuhrer and Parmentier, 1977; Bogs and Lenhardt, 1971). Other intermediates for the formulation of multiparticulate systems are granules and pellets (Ghebre-Selassie, 1994). Moisture and heat can damage PAN, and accordingly the application of wet granulation must be considered carefully (Thoma and Bechtold, 1999). The use of melt granulation is also questionable since enzymes can then undergo thermal damage.

PAN is digested in the gastric juice and acts in the ileum, and an intestinosolvent coating is therefore necessary (Thoma and Bechtold, 1999). Accordingly, the optimization process for the dosage form containing this component must involve the coating step. We previously studied the properties of the surface of tablets containing PAN. The surface enrichment of PAN was detected on tablets containing microcrystalline cellulose as a conventional binder (Kristó et al., in press). Investigation of the influence of moisture on the starch-hydrolysing activity of PAN is important, because the possibility of modification is higher in response to liquids.

Our present aims were therefore to study the effects of direct compression, granulation and coating on the starch-hydrolysing activity of PAN. The starch-hydrolysing activity of PAN was investigated during the modelling of circumstances of direct compression and wet granulation. Measurements of starch-hydrolysing activity were made according to Ph. Eur. In the first part of this study, the effects of direct compression were evaluated. Meat is generated during direct compression. The thermal stress of the dry material was studied separately, with the objective of an evaluation of the behaviour of this component in the possible "hot spots". Additives were not applied in this study; on the other hand, the effects of interactions on the starch-hydrolysing activity should be taken into consideration during tabletting. In the second step, the simultaneous effects of moisture and elevated temperature on the starch-hydrolysing activity of PAN were tested by means of a factorial design. Combinations of these factors can occur during granulation and coating. Aqueous and ethanolic coating liquids are typically used for the surface treatment of the solid dosage form (Cole et al., 1995; Bauer et al., 1988), and thus these liquids were applied. This approach for the assessment of the effects of different factors is not conventional for enzyme-containing dosage forms. This information can broaden the understanding of the effects of different technological processes, which is inevitable for the determination of the critical control point in the preparation of solids containing proteins. Its relevance is constantly increasing because of the spreading of biotechnology and protein-type active agents.

2. Materials and methods

2.1. Materials

Pancreatin (PAN) (Ph. Eur. Richter Gedeon Plc., Budapest, Hungary) (starch-hydrolysing activity: 18 EPU/mg; proteolytic activity: 3.6 EPU/mg; lipolytic activity: 41 EPU/mg; fat content: 2.2%) was applied as active agent. The moisture content of the untreated PAN was 6.68%. Water-soluble starch (Spektrum-3D, Debrecen, Hungary) was applied for measurement of the starch-hydrolysing activity. Distilled water and 96% ethanol (Spektrum-3D, Debrecen, Hungary) were applied for the study of the effects of wet conditions.

2.2. Measurement of starch-hydrolysing activity

The starch-hydrolysing activity of PAN was measured according to Ph. Eur. The concentration of the iodine-starch complex was determined at 576 nm with a UV spectrophotometer (Unicam Helios Alpha, Spectronic Unicam, UK). The amount of starch hydrolysed was calculated via the amount of the iodine–starch complex. The measurement was performed in consecutive steps. The first mixture contained 1250 μl 2% aqueous solution of starch, 500 μl pH 6.8 phosphate buffer, 50 μl 11.7 g/l sodium chloride solution and 50 μl PAN solution. It was incubated for 10 min at 37 °C in a water bath. After this, 100 μl 1 M hydrochloric acid, 500 μl 0.05 M iodine solution containing potassium iodide, and 2250 μl 0.1 M sodium hydroxide solution were added, the mixture was left to stand for 15 min at room temperature, and finally 200 ml diluted sulphuric acid (20%) was added. The starch-hydrolysing activity was determined; the starch-hydrolysing activity of the untreated PAN was taken as 100%.

2.3. Determination of effect of compression

The starch-hydrolysing activity was investigated during the modelling of the circumstances of tabletting. Comprimates 12 mm in diameter were prepared with a hydraulic press (Specac Inc., Graseby) at loads of 2, 4, 6, 8 or 10 t (19.62, 39.24, 58.86, 78.48 or 98.1 kN). The surface of the comprimates was flat. The resulting tablets were pulverized in a mortar before the starch-hydrolysing activity testing. Not only the effect of the pressure was examined. High pressure is known to induce the generation of heat. This occurs as a very rapid phenomenon during compression. For clarification of this situation, elevated temperatures (40, 50, 60, 70, 80, 90 and 100 °C) were applied in independent tests. In order to study the starch-hydrolysing activity during elevated temperature, the untreated PAN was stored for 2 h under dry air conditions in a thermostat (Hereaus Instruments, Hanau, Germany) in which the heat was distributed homogeneously.

2.4. Determination of effects of wet conditions

The 2^3 full factorial design was applied with 2 central points (Statistica for Windows) to evaluate the effects of the factors on the starch-hydrolysing activity of PAN. The factors investigated were temperature, time and liquid content (Table 1). The liquid was ethanol or distilled water. Homogeneous mixtures were prepared in a mortar and the resulting wet masses were stored in hermetically closed containers for a given time. The amount of liquid added to the powder is given as a percentage of the mass of wet mass. The content of liquid utilized during wet granulation is ~30–60%; hence, this range of liquid content was applied in these evaluations. During the calculation of the starch-hydrolysing activity, the exact liquid contents were considered.

The following approach, involving the interactions of the factors, was used to determine the response surface and the

Table 1 – Values of factors.

Factor	Low level (−)	Zero level (0)	High level (+)
Temperature (x_1)	40 °C	50 °C	60 °C
Time (x_2)	1 h	1.5 h	2 h
Content of liquid (x_3)	30%	45%	60%

Table 2 – Starch-hydrolysing activity of PAN treated by compression.

Load (t)	Activity (%)
2	98.87 ± 0.03
4	98.84 ± 0.13
6	98.61 ± 0.09
8	98.52 ± 0.19
10	98.61 ± 0.11

relative effects of the factors (b):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3$$

Statistica for Windows 8.1 AGA software (StatSoft, Inc. Tulsa, USA) was employed for the calculations. During the mathematical evaluations, the confidence interval was 95%, i.e. the differences were significant if $p < 0.05$.

3. Results and discussion

In the first step, the effect of the compression force on the starch-hydrolysing activity of PAN was determined. There was no significant alteration in this parameter: a slight decrease in starch-hydrolysing activity was detected, but it was negligible. The heat generated during compression can induce the degradation of this active component. Its incidence and extent can depend on the tablet-making parameters and on the properties of the tablets. This problem was not detected in our study (Table 2). To investigate the effects of elevated temperature on the starch-hydrolysing activity of PAN, independent thermal-stress tests were performed. In order to study the starch-hydrolysing activity during elevated temperature, the untreated PAN was stored for 2 h under dry air conditions in a thermostat (Hereaus Instruments, Hanau, Germany) in which the heat was distributed homogeneously. No significant effect was observed, and it can therefore be concluded that direct compression can be applied for the processing of PAN, since neither the pressure nor the temperature induced any appreciable modification of its function (Table 3).

In the second step, the effects of wet conditions were tested. Close to 100% starch-hydrolysing activity was detected for some of the samples treated with water at low temperature, whereas the highest value was nearly 70% for the samples wetted with ethanol. It is also to be seen for both liquids that special combinations of factors can induce a starch-hydrolysing activity of only 7% (Table 4).

For the exact evaluation of the effects of the factors, linear fitting was applied in both cases (Table 5). The b_0 value was ~54% lower for ethanol than for water. Since this is a mean value, it can be concluded that in all cases PAN is more sensitive to ethanol than to water. Only temperature was a significant factor for both liquids; its negative effect was ~60% higher for ethanol than for water. In this case, the other factors did not cause significant change. The content of liquid was at least 30% (this range is typically used during conventional granulation), and thus the maximum effect can be achieved, so that further increase of this component cannot induce a higher decrease of the starch-hydrolysing activity of PAN. High temperature did not cause a decrease in the starch-hydrolysing activity of relatively dry PAN (untreated PAN with a ~7% moisture content). Conventional granulation/pelletization can therefore induce a decrease in the starch-hydrolysing activity of PAN when elevated temperature and high liquid content are applied at the same time. Optimization of the moisture content in the range between ~7% (the moisture content of untreated PAN) and 30% is not necessary, as a liquid content under 30% is not appropriate for the preparation of granules by wet granulation. The other explanation for the neglect of this step is the fact that topical overwetting cannot be avoided during the wetting in the granulator. The homogeneous distribution of the wetting liquid is a time-consuming step, and during this process a higher amount of liquid can therefore be detected in certain microvolumes of the mass. Accurate determination of the effect of a difference of a few per cent in the moisture content is difficult.

Time and its interaction with temperature exhibited non-significant, but considerable effects for ethanol ($b_{13} = 8.32$). The two factor interactions were irrelevant for water.

Table 3 – Starch-hydrolysing activity of PAN treated by elevated temperature.

Temp. (°C)	Activity (%)
40	100.01 ± 0.04
50	99.62 ± 0.18
60	99.54 ± 0.02
70	99.69 ± 0.08
80	99.65 ± 0.09
100	99.60 ± 0.13

Table 4 – Starch-hydrolysing activity of PAN treated by wetting.

Temp. (°C)	Time (h)	Content of liquid (%)	Activity – water (%)	Activity – ethanol (%)
40	1	30	99.04 ± 0.23	47.28 ± 2.23
40	1	60	98.61 ± 0.01	67.55 ± 2.67
40	2	30	98.98 ± 0.15	19.15 ± 4.08
40	2	60	84.44 ± 1.53	30.62 ± 0.78
60	1	30	23.63 ± 3.31	7.07 ± 2.09
60	1	60	6.91 ± 3.02	7.9 ± 0.92
60	2	30	11.08 ± 1.83	8.85 ± 2.71
60	2	60	9.17 ± 1.71	7.65 ± 3.25
50	1.5	45	28.11 ± 2.81	15.84 ± 3.6
50	1.5	45	26.78 ± 2.48	11.65 ± 1.08

Table 5 – Effects of factors.

Factor	Coefficient for water	Coefficient for ethanol
R^2	0.9253	0.9481
b_0	48.67	22.36
b_1 (temperature)	−41.29*	−16.64*
b_2 (liquid content)	−4.2	3.92
b_3 (time)	−3.06	−7.95
b_{12}	−0.46	−4.02
b_{13}	0.49	8.32
b_{23}	0.09	−1.35
b_{123}	3.62	0.85

R^2: coefficient of determination; b_0: mean value; b_1, b_2, b_3: main effect; b_{12}, b_{13}, b_{23}: two factor interaction; b_{123}: three factor interaction.

* Significant ($p < 0.05$).

4. Conclusion

It can be concluded that the starch-hydrolysing activity of PAN was not changed because of direct compression. High temperature did not induce a decrease in this parameter for the relatively dry active component. Modelling of the wet conditions which can occur during granulation and coating led to significant modifications. Ethanol caused more relevant changes than water. In both cases, the most important factor was temperature. At 40 °C, the starch-hydrolysing activity was not altered significantly for the sample wetted with water. The effects of the liquid content in the range 30–60% and the time interval 1–2 h were negligible. An increased liquid content was necessary for an undesirable effect, but the change in the amount was not highly relevant.

This information is helpful as concerns the design of the preparation of multiparticulate dosage forms containing PAN. Wet granulation is not the most appropriate method for the preparation of an intermediate. It can be applied if the temperature is <40 °C, only a low amount of water is applied, and the process time is short. A better way is to produce minitablets by direct compression, since degradation of the active agent can then be avoided. Wet conditions must also be considered during the coating. This step is obligatory in this case and ethanol cannot be used as liquid. Aqueous systems must be applied at low temperature, but quick drying is also advisable, since overwetting of the surface can occur. The additional operational parameters applied during the surface treatment must therefore be optimized for the quick drying of the first layer of the film (e.g. using slow atomization with a high amount of drying air in the first phase, and later the rate of atomization can be enhanced). Such preformulation studies can reveal the importance of the different parameters, and are necessary for optimization of the preparation of dosage forms containing sensitive proteins. These results are important when the aim is the formulation of two protein active agents into a multiparticulate solid dosage form, because proteins are very sensitive materials. Maintenance of the activity of PAN and the other protein should be taken into consideration during formulation. This study emphasizes the importance of special aspects in the process of solid dosage forms containing proteins.

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