

Formulation of apigenin containing multiparticulate drug delivery systems

PhD thesis

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Introduction

The epidemiological observation that a variety of degenerative illnesses can be prevented by consuming vegetables, fruits and medicinal herbs has been scientifically proved. Such pathological changes include chronic inflammation, cardiovascular diseases and malignant tumors where also the accumulation of free radicals plays a significant role. Consequently, plants with bioactive compounds have a salutary effect on the human organism especially because of their antioxidant capacities. Apigenin is one of the notable bioactive flavonoids, with its capacity of oxygen radical absorbance and decrease of chronic inflammation. Thus, it prevents the formation of cardiovascular diseases and tumors combined with its anti carcinogenic properties as it is able to affect cell signal transduction. In recent years medical science has adopted a new attitude, emphasizing the crucial importance of prevention. Different herbal products and products having a medicinal effect play a significant role now in prevention as well as during the treatment and follow-up care processes. Therefore, phytocomponents also attract a growing interest from drug research and development both for their outstanding effects and low risk of side effects. Another important advantage is that producing them can be more cost-effective, since the development of a new active substance produced by synthesis is remarkably costly and time-consuming.

Similarly to synthetic pharmaceutical molecules, water solubility and permeability play an important role in the efficacy of the product. Ensuring the adequate dissolution and absorption and accordingly the necessary concentration is essential in the development process of the product. Apigenin was classified as class II drug of the Biopharmaceutical Classification System (BCS) due to its low solubility and high permeability, consequently one of the main goals of its formulation is to improve its solubility. Since the pharmaceutical industry has an ongoing demand for always new and more effective processes in the pharmaceutical chemistry and technology, developing an innovative product high in apigenin which may as well ensure controlled active substance release could considerably contribute to the success of drug therapy.

Aims

The main objective of my doctoral thesis were to develop an optimal drug delivery system for apigenin which could contribute to therapy. Therefore my aims were as follows:

- *Analytical method* development for determining apigenin in the herbal extract and the total apigenin concentration of the formulations.
- *Solubility improvement of apigenin with parent cyclodextrins and β -cyclodextrin derivatives* in different physiological pH values. Investigation of the stoichiometry and the apparent stability constants of the complexes and determining antioxidant activity.
- Formulation of multiparticulate drug delivery system for apigenin by using inert pellet cores. Optimizing the layering and enteric polymer coating conditions of the fluidization process and monitoring it by FTIR and NIR instruments.
- Physical characterisation of the developed *apigenin containing pellets with modified drug release*, such as particle shape and size, particle size distribution, crushing strength as well as the dissolution of apigenin *in vitro*.
- Preparation of apigenin-loaded biocompatible *serum albumin nanoparticles* and characterization in terms of size, zeta potential and drug loading, additionally the fluorescence properties were investigated.
- Spray drying of nanoparticles without excipient, conventional (carrier excipient) and new generation (carrier free) excipients. Investigation of the aerodynamic properties of the dry powders by next generation cascade impactor *in vitro*.

Methods

Solubility improvement of apigenin with cyclodextrins

The phase-solubility studies were performed according to the method of Higuchi and Connors. Experimentally, fixed excess amounts of apigenin were added into capped vials which contained a constant volume (2 ml) of distilled water and buffer solutions (pH 1.0 and pH 6.8) with increasing concentrations of each cyclodextrins (CDs, 1.0–50.0 mM). The vials were vortex mixed for 30 sec, stored at constant room temperature (25°C) and protected from light to prevent degradation. After reaching equilibrium for solubilization (72 h), each vial was centrifuged at and the sample was taken from the supernatant and filtered through 0.45

µm membrane filter. The total apigenin concentration of each sample was determined in triplicate by HPLC-UV method (Agilent 1100, Agilent Technologies Inc., USA).

The apparent stability constants ($K_{1:1}$) of Api-CD complexes were calculated from the phase solubility diagrams obtained according to the Higuchi and Connors method, assuming that one guest molecule (Api) forms a complex with one cyclodextrin (CD) molecule:



$$K_{1:1} = \frac{[\text{ApiCD}]}{[\text{Api}][\text{CD}]} \quad (2)$$

$$K_{1:1} = \frac{\text{slope}}{\text{intercept}(1-\text{slope})} \quad (3)$$

Preparation of apigenin layered pellets with modified release

The optimized extraction procedure was performed to achieve a parsley extract with high apigenin content. Thereafter the ethanol from the obtained extract was evaporated under vacuum at 60 °C (Büchi Rotavapor R-200, Büchi Labortechnik AG, Switzerland). The aqueous extract was further lyophilized (Finn-Aqua Lyovac GT3, Germany) and redissolved in aqueous HPMC solution (Pharmacoat® 606; 2.0 % w/w) and layered onto the inert cores with in a centrifuged bottom spray configured fluidized bed apparatus (Aeromatic Strea I, Aeromatic-Fielder AG, Switzerland) to achieve 5 mg apigenin content / 1 g pellet. During the layering process, the dispersion was stirred continuously at room temperature. Under similar conditions, Apigenin powder was layered to inert cores to achieve 20 mg / 1 g pellet. The layered cores were divided into two parts: the first part was coated with Eudragit® L 30 D-55 and the second part with Eudragit® FS 30 D using the same fluid bed apparatus described previously. The concentration of film forming polymer in the coating suspension was 25.8 % w/w. TEC (10%, w/w on dry Eudragit® L 30 or 5 %, w/w on dry Eudragit® FS 30) and micronized talc (< 10 µm, 50% w/w on dry polymer) were used. During the coating process in order to prevent the sedimentation of the talc, the suspensions were stirred continuously. A series of film coated herbal extract loaded pellets were produced with different polymer film thicknesses and quantified by the weight gain (5% w/w; 10% w/w and 15% w/w). The preparation procedure was the same at both cases. The optimized layering and coating conditions for the preparation of pellets in fluid bed process parameters can be seen on **Table I**.

TABLE I. OPTIMIZED LAYERING AND COATING CONDITIONS OF FLUIDIZATION

Parameters	Layering	Coating	
		Eudragit® L 30	Eudragit® FS 30
Batch size (g)	300	150	150
Spray nozzle diameter (mm)	1.2	1.2	1.2
Inlet air temperature (°C)	45-47	39-41	28-31
Outlet air temperature (°C)	35-39	34-36	25-26
Atomizing pressure (bar)	0.8	0.8	0.8
Fluid air flow rate (m ³ /h)	80-120	80-100	80-100
Spray rate (g/min)	5-7	5-10	2-4
Drying temperature (°C)	45	45	30
Drying time (min)	15	15	15

Preparation of albumin nanoparticles and spray drying

BSA nanoparticles were prepared using a nanoparticle albumin bound technology with minor modifications²⁹. Briefly, 1000 mg of BSA was dissolved in 50 ml of distilled water saturated with chloroform. Separately, 100 mg of Api was dissolved in 3 ml of chloroform saturated with water and ultrasonicated for 10 minutes. These two solutions were mixed and ultrasonicated for 20 minutes with a probe-type sonicator (MSE Soniprep 150 Ultrasonic Processor, MSE Ltd., London, UK) on ice. After homogenization, the chloroform was evaporated by rotary evaporator at 25°C for 15 minutes. The obtained nanoparticles were filtered (0.45µm) and further spray dried. Spray drying of the BSA-Api formulations without excipient and in the presence of lactose monohydrate (50 %, w/w) and L-leucine (9%, w/w) were carried out in a Büchi 290 Mini Spray Dryer (BÜCHI Labortechnik AG, Flawil, Switzerland). The operating conditions are presented in **Table II**. The powders were collected from the lower part of the cyclone and the collecting vessel, stored in tightly sealed glass vials under vacuum at room temperature.

TABLE II. OPTIMIZED CONDITIONS OF SPRAY DRYING

Parameters	Values
Nozzle diameter (mm)	0.1
Inlet temperature (°C)	120
Outlet temperature (°C)	65-70
Drying airflow (l/h)	600
Aspiration rate (m ³ /h)	35
Liquid feed rate (ml/perc)	5

Study on antioxidant activity

The free radical scavenging activities of the samples were measured by using DPPH• stable free radical with a characteristic absorbance peak at 517 nm. Briefly, methanolic stock solution of 0.1 mM DPPH• reagent was freshly prepared and protected from light. A standard curve was plotted between the DPPH• concentration and absorbance. The linear relationship was calculated graphically ($R^2 > 0.999$). The exact concentration of the free radical was calculated using the standard curve. 1 ml of the samples was added to 2 ml of 0.06 mM DPPH• methanolic solution, vortex mixed for 10 seconds and protected from light. The addition of samples resulted in a decrease in the absorbance due to the scavenging activity of the oxidisable groups of antioxidants. The absorbance at 517 nm was determined in every 15 min until the steady state, 0.1 mg/ml apigenin stock solution was used as a standard.

$$I (\%) = \frac{A_0 - A_s}{A_0} \times 100 \quad (4)$$

The antioxidant activity was expressed as % DPPH• remaining in the solution (I, %), A_0 is the absorbance of the DPPH• stock solution and A_s is the absorbance of the sample. A lower concentration of remaining free radicals corresponds to a stronger antioxidant. All measurements were carried out in triplicates.

Results

Solubility improvent of apigenin with cyclodextrins

- The solubility improvement of apigenin with broad concentrations of cyclodextrins in physiological pH values was investigated for the first time based on literature data. According to the phase-solubility studies, in case of HP- β -CD

and SBE- β -CD, 1:1 Api:CD inclusion complexes were formed. However, the inclusion complex formation with γ -CD and RM- β -CD and suggesting a higher-order complexes (eg. 1:2 Api:CD).

- It was concluded that the presence of charge and different buffer aqueous media have impact on the complexation of apigenin as well. Besides the release of the enthalpy-rich water from the cavity of CD, other forces like H-bond, van der Waals, ionic strength, ring strain and solvent-surface tensions also contribute.
- The most effective complex forming agent proved to be the RM- β -CD, where 150-fold increase could be achieved in the solubility of apigenin in pH 6.8 buffer (~0,230 mg/ml).
- The inclusion complex altered the spectral properties of apigenin according to UV-vis and spectrofluorimetric measurements, incorporation of a phenolic chromophore group of apigenin in the cyclodextrin cavity and secondary bonding forces could be assumed.
- The studies on antioxidant properties pointed out the enhanced scavenging effect of the encapsulated apigenin due to the improved concentration. The enhanced hydrogen donating ability can be hypothesized as well.

Apigenin layered pellets with modified release

- Layering of apigenin and apigenin containing herbal extract to inert cores as well as the coating process with Eudragit[®] L and Eudragit[®] FS polymers were optimized in fluid bed apparatus.
- Microcrystalline cellulose was proved to be suitable carrier for apigenin and the herbal extract with high apigenin content (~20 mg apigenin /1 g pellet and ~5 mg apigenin in herbal extract /1 g pellet).
- The produced layered and coated pellets have adequate physical properties such as particle size and shape, particle distribution and crushing strength, moreover, the good flowability properties of the starter core was not altered therefore good packing into tablets or capsules can be assumed.
- The apigenin release from the layered cores was immediate regardless of the pH values.
- 10 % (w/w) Eudragit[®] L 30 and 15% (w/w) Eudragit[®] FS 30 polymer content ensured the site-specific release of apigenin.
- The dissolution of water soluble apigenin glycosides was faster compare to the apigenin aglycon at both cases.

- The antioxidant study provided evidence that both formulations are effective *in vitro*.

Apigenin loaded serum albumin nanoparticles for targeted release

- Due to the high encapsulation efficiency ($82.61 \pm 4.56 \%$), the small particle size ($376 \pm 7.824 \text{ nm}$) and adequate zeta potential ($-19.20 \pm 0.818 \text{ mV}$), serum albumin is a suitable biodegradable carrier for apigenin ($\sim 1.7 \text{ mg apigenin /ml}$).
- Fluorescence studies proved for the first time that apigenin is binded to the subdomain II A of albumin nanoparticles.
- Spray drying of nanoparticles yielded dry powders with low moisture content and optimal aerodynamic properties *in vitro*. Therefore the use of excipients may not required. The new generation excipient (L-leucin) was more effective than conventional lactose monohydrate ($\sim 2 \text{ mg apigenin/ dose}$).
- The radical scavenging activity of apigenin was maintained in the formulations.

Conclusions

The formulation of active ingredients from biological sources is challenging. The aims of my work were to increase the aqueous solubility of apigenin and to develop an innovative carrier system. Cyclodextrins were applied to increase water solubility. I prepared apigenin-cyclodextrin inclusion complexes and concluded that the derivatives was more effective than parent cyclodextrins. As the result of the inclusion complex formation, the scavenging activity of apigenin has been significantly improved due to the improved concentration and H-donor activity.

Oral delivery of apigenin containing pellets with different polymer coatings have been developed to achieve modified release. Herbal extract as a natural source of apigenin (*Petroselinum crispum*) has been also used to increase the antioxidant activity due to the synergistic effects of the compounds. Therefore the enteric coatings allow increased concentration at the absorption site in the duodenum and the colon which can lead to enhanced bioavailability. The improved apigenin content makes possible higher intake therefore local and systemic health benefits as well.

As alternative dosage I created a dry-powder inhaler system where apigenin is binded to serum albumin nanoparticles. *In vitro* optimal aerodynamic properties and therefore targeted delivery could be ensured by spray drying the nanoparticles without excipient as well. The antioxidant activity was not hindered.

List of publications

Papers related to the Ph.D. thesis

Book chapter

1. Pápay ZsE, Balogh E, Zariwala GM, Somavarapu S, Antal I. *Drug Delivery Approaches for Apigenin: A Review*. In Nilus M Stacks (szerk.), *Apigenin and Naringenin: Natural Sources, Pharmacology and Role in Cancer Prevention*. Nova Science Publishers, Hauppauge, 2015: 1-20. (ISBN:978-1-63463-987-3)

Journal Papers

2. Pápay ZsE, Kállai-Szabó N, Ludányi K, Klebovich I, Antal I. (2016) *Development of oral site-specific pellets containing flavonoid extract with antioxidant activity*. Eur J Pharm Sci, 95: 161-169.
3. Pápay ZsE, Kállai-Szabó N, Balogh E, Ludányi K, Klebovich I, Antal I. (2016) *Controlled release oral delivery of apigenin containing pellets with antioxidant activity*. Curr Drug Deliv, 13: 1-10.
4. Pápay ZsE, Sebestyén Z, Ludányi K, Kállai N, Balogh E, Kósa A, Somavarapu S, Böddi B, Antal I. (2016) *Comparative evaluation of the effect of cyclodextrins and pH on aqueous solubility of apigenin*. J Pharm Biomed Anal, 117: 210-216.
5. Pápay ZsE, Sornsute A, Merchant Z, Antal I, Somavarapu S. (2014) *Rossz vízoldhatóságú flavonoidok oldékonyság növelésének lehetőségei*. Gyógyszerészet 87: (Suppl.1) S113.
6. Pápay ZsE, Antal I. (2014) *Study on the antioxidant activity during the formulation of biological active ingredient*. ESJ, 3: 252-257.
7. Pápay ZsE, Kósa A, Boldizsár I, Ruszkai Á, Balogh E, Klebovich I, Antal I. (2012) *Petroselinum crispum kivonatának gyógyszerészeti vonatkozásai és formulálási lehetőségei*. Acta Pharm Hung, 82: 3-14.

Other publications

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9. Antal I, Budai M, Dávid Á, Kállai N, Klebovich I, Ludányi K, Marton S, Mike-Kaszás N, Pápay ZsE, Plachy J, Sebestyén Z. *Kémiai ellenőrző vizsgálatok a gyógyszertechnológiában: Egyetemi jegyzet IV. éves gyógyszerészhallgatók részére*. Antal István, Klebovich Imre, Ludányi Krisztina (szerk.), Semmelweis Kiadó, Budapest, 2012: 169. (ISBN:9789633312582)