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5-ASA loaded chitosan–Ca–alginate microparticles: Preparation and physicochemical characterization

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Abstract

The objective of the work was to prepare chitosan–Ca–alginate microparticles that can effectively deliver 5-ASA to the colon after peroral administration. For these requirements, a spray-drying technique was applied to 5-ASA/sodium alginate aqueous solution to obtain spherical particles having a mean diameter less than 10 μm. The microparticles formed were cross-linked and coated into solution of $CaCl_2$ and chitosan to obtain stable microsystem. ^{1}H NMR and UV–vis spectra of 5-ASA have shown no degradation when working in adequate conditions, such as light protection, freshly prepared solution and use of nitrogen to prevent the oxidative self-coupling of 5-ASA moieties. By imaging with SEM, acceptable spherical morphology was observed, but also flattened, disk-shaped particles of smooth surface and low porosity. CLSM imaging showed dominant localization of chitosan in the particle wall, while for alginate, a homogeneous distribution throughout the particle was observed giving the particles negative charge. In the FTIR spectra of 5-ASA loaded Ca–alginate microparticles the characteristic peaks of 5-ASA were not altered indicating no covalent interaction between the drug and the polymer. DSC and X-ray diffraction studies revealed that 5-ASA was molecularly dispersed within the chitosan–Ca–alginate microparticles during the production process.

Keywords: Alginate; Chitosan; 5-ASA; Microparticles; Spray-drying method; Physicochemical characterization

1. Introduction

There has been increasing interest in the study of alginate and chitosan in connection with various applications mainly for their non-toxic, biocompatible, biodegradable and mucoadhesive properties. They have shown their potential for use as scaffolds in tissue-engineered medical products (Pariente et al., 2001), as an encapsulating matrix for immobilization of living cells (Iyer et al., 2005; Krasaekoopt et al., 2006) and as drug delivery systems (Tozaki et al., 1999, 2002; Giunchedi et al., 2002; Hejazi and Amiji, 2003). Among the various drug delivery systems investigated to achieve efficient and site-specific delivery microparticles have received considerable attention (Li et al., 2002; Wittaya-areekul et al., 2006; Ye et al., 2006).

To date, the production of alginate gel microparticles has been accomplished mainly by interfacial cross-linking reaction with external or internal calcium source (Fundueanu et al., 1999; Vandenberg and Noue, 2001; Liu et al., 2002; Ribeiro et al., 2005). This procedure has been used to encapsulate a wide variety of low molecular drugs (Acarturk and Takka, 1999; Takka and Acarturk, 1999; El-Kamel et al., 2003), proteins (Coppi et al., 2002), growth factors (Gu et al., 2004), DNA (Mittal et al., 2001) and hemoglobin (Huguet and Dellacherie, 1996). The main disadvantages using this procedure include complicated

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industrial scale-up (Poncelet et al., 1992), inhomogeneous physical hydrogels (Hoffman, 2001) and difficulties in obtaining lower size microparticles, which are preferred for stabilizing the fragile structure of the drug and controlling delivery kinetics. Coppi et al. (2001, 2002) investigated the feasibility of the spray-drying technique to produce less than 10-μm microparticles of alginate cross-linked by calcium ions for the transport of bovine serum albumin as a model protein. The microparticles were of appropriate size and immobilization capacity and the stability of the protein to preparative conditions was also confirmed.

Coating alginate microparticles with polycationic polymers, such as chitosan, has been commonly used to control the disintegration of alginate microparticles and extend the drug release. Usually, chitosan–alginate microparticles are obtained by two principally different procedures: a one-step method, where a complex coacervate membrane is formed at the interface between the alginate and chitosan solutions when the alginate solution is dropped directly into a solution of calcium chloride mixed with chitosan. The other method is a two-step method, where Ca–alginate microparticles are recovered and subsequently coated with chitosan (Gaserod et al., 1998, 1999). Chitosan–alginate complex erodes slowly in phosphate buffer at pH values higher than 6.5 suppressing the initial drug release in the upper segments of the intestine occurring for uncoated microparticles and controlling the release in the colon whereas pH value is in the range of 6.5–7.0 (Tapia et al., 2004). This behavior and degradation of chitosan by the microflora available in the colon makes chitosan–alginate microparticulated system a good candidate for effective drug delivery in the treatment of inflammatory bowel diseases (IBD) after oral administration.

In the case of IBD, an enhanced uptake of administered particles by neutrophils, natural killer cells, mast cells and regulatory T cells in the inflamed tissue was observed (Lamprecht et al., 2001a). It has been also reported that microparticles can be efficiently taken up by macrophages allowing accumulation of the particulate carrier systems in the desired area (Tabata et al., 1996; Stein et al., 1998). A size-dependent particle deposition in the inflamed area of the colon was observed with increased adherence of particles with a nominal size between 100 and 10 μm (Lamprecht et al., 2001b). Charge interactions were reported to further enhance binding to the inflamed tissue. Negatively charged particles adhered more readily to the inflamed tissue due to the high concentrations of positively charged proteins that increase the affinity to negatively charged substances. This was confirmed in the study of Bernkop-Schnurch et al. (2001), in which anionic alginate showed more potent mucoadhesion in comparison with the cationic chitosan. Consequently, an increased residence time at the inflammation site can be postulated for negatively charged chitosan–Ca–alginate microparticles. With chitosan localization in the particle wall controlled release in the colon could be achieved. This should allow a dose reduction and also side effects could be significantly minimized in the case of conventional chemical anti-inflammatory compounds.

Considering above-mentioned, the aim of the study was to prepare chitosan–Ca–alginate microparticles for prolonged and colon-specific delivery of 5-aminosalicylic acid (5-ASA), which is a commonly used drug in the treatment of IBD. For these requirements, a spray-drying method associated with polymer complexation/gelation (Coppi et al., 2001; Liu et al., 1997) was used in order to obtain particles less than 10 μm. Physicochemical characterization, including microparticle size, morphology, polymers distribution, zeta potential, drug content and drug–polymers interaction was performed as a function of the preparation procedure. In particular, 5-ASA stability during microparticle preparation and in simulated *in vivo* conditions was evaluated.

2. Materials and methods

2.1. Materials

Sodium alginate (LF 10/60, fG 65–75%, viscosity, 20–70 mPa s for an aqueous solution, 1%, w/v) was purchased from Protanal FMC BioPolymers (Dramen, Norway). Chitosan as a free base (150 kDa, $[R_G^2]^{1/2}$ 44 ± 5 nm, viscosity 20–100 mPa s for 1% (w/w) solution in 1% (w/w) acetic acid, minimum 85% deacetylated was donated by France Chitine (Marsey, France). 5-ASA was purchased from Fluka Chemie AG (Switzerland). Calcium chloride and fluoresceine 5(6)-isothiocyanate, mixed isomers (FITC), were supplied from Sigma–Aldrich, Inc. (St. Louis, MO, USA), while rhodamine-B-isothiocyanate, RBITC, from Fluka Chemie AG (Steinheim, Germany). All other reagents were of analytical grade.

2.2. Preparation of microparticles

5-ASA loaded chitosan–Ca–alginate microparticles were prepared with slight modifications of the spray-drying method associated with polymer complexation/gelation (Coppi et al., 2001; Liu et al., 1997). Namely, aqueous dispersion (30 ml) of alginate (3%, w/w) and 5-ASA (0.5%, w/w), adjusted to pH 7.0 by 0.2 M NaOH was infused into a spray dryer nozzle unit of Büchi Mini Spray Dryer B-191 (Büchi Laboratorius-Technik AG, Flawil, Switzerland) and continuously sprayed using an automatic infusion/withdrawal pump (model Sonceboz 3.1 A/pH, Switzerland) into 90 ml solution of chitosan (0.25%, w/w) and $CaCl_2$ (2.5%, w/v) in 1% (w/w) acetic acid, which was placed in the apparatus collector.

The conditions of the spray-drying process were: nozzle diameter 0.7 mm, aspirator pressure 100%, atomizer pressure 600 Nl h^{-1}, flow rate 10 ml/min, inlet temperature 140 °C, outlet temperature 100 °C. The dispersion of microparticles was collected and they were allowed to harden under stirring (3000 rpm) for at least 4 h at room temperature. The microparticles thus formed were separated by centrifugation at 3000 rpm for 15 min (Jouan B 3–11, France), washed with distilled water and freeze-dried at 0.250 mbar and −50 °C for 24 h (Bioblock Scientific Christ Alpha 1–4, Germany). Blank alginate–, Ca–alginate– and chitosan–Ca–alginate microparticles were prepared using the same conditions as for the drug-loaded particles. 5-ASA loaded alginate microparticles (termed as “temporary” microparticles) were also prepared by spray-drying of aqueous dispersion of

alginate and 5-ASA using the same formulation and spray-drying conditions.

2.3. Stability tests of 5-ASA in solutions and in microparticles

Stability tests of 5-ASA were performed on 5-ASA solutions at various pHs: 3.0 (0.5%, w/w in solution of 1%, w/w acetic acid), 5.0 (aqueous solution of 5-ASA, 0.5%, w/w), 7.0 (aqueous solution of 5-ASA, 0.5%, w/w, with pH adjusted by 0.2 M NaOH) and 7.4 (solution of 5-ASA in phosphate buffer saline, PBS, 0.5%, w/w). The solvents and pH range were chosen to simulate the preparation and drug release conditions. ^{1}H NMR spectra in H_2O/D_2O (90/10, Bruker Advance DRX 500, Germany) and UV–vis spectra (UV/VIS spectrophotometer, Perkin–Elmer Lambda 16, USA) of 5-ASA in a range of 200–750 nm were recorded in different time intervals during 1 week. All the samples tested were light-protected and nitrogen was used in solutions to prevent the oxidative self-coupling of 5-ASA moieties. In order to evaluate the stability of 5-ASA in microparticles, ^{1}H NMR and UV–vis spectra of 5-ASA were recorded after 5-ASA release from alginate– and chitosan–Ca–alginate microparticles in PBS (pH 7.0–7.4). Microparticles and drug release mediums were light-protected and CO_2 in drug release mediums was added in order to maintain an anaerobic environment.

2.4. Characterization of microparticles

2.4.1. Particle size analysis

Measurement of the particle size and diameter of the freeze-dried microparticles was carried out with Beckman Coulter Multisizer (Coulter, USA) after suspending the particles in conducted liquid Isoton containing Tween 80, 1% (w/w) and sonicating the suspension for 10 min in ultrasonic bath.

The average particle size was expressed as the volume surface diameter, d_{vs} (μm) (Edmundson, 1967).

2.4.2. Zeta potential

Suspension of microparticles in PBS diluted (0.0001 M, pH 6.8) previously sonicated for 10 min in US bath was used as a medium to determine the zeta potential of blank and 5-ASA loaded chitosan–Ca–alginate microparticles. The pH value was chosen to simulate the pH value of the colon. The zeta potential of the particles was recorded using Malvern Zeta Sizer 2000 (Malvern Instruments Ltd., UK). Each sample was analysed at least six times to obtain an average value and a standard deviation. Samples of blank Ca–alginate microparticles were also submitted to this study.

2.4.3. Content of 5-ASA in the microparticles

To evaluate the actual content of 5-ASA in the chitosan–Ca–alginate microparticles, at first, drug content in "temporary" microparticles was determined by dissolving an exactly weighed amount of microparticles in PBS pH 7.0 with magnetic stirring at room temperature under light and oxygen protection until complete dissolution. The yield of the spray-drying process was 55 ± 19%, calculated as an average value from five determinations. These values were particularly satisfying knowing the generally low production yield encountered with laboratory scale spray-dryers (Giunchedi et al., 1996; De Jaeghere et al., 2001).

Real drug content was calculated as amount of 5-ASA in the microparticles in respect to the real amount of the chitosan–Ca–alginate microparticles obtained after freeze-drying (Coppi et al., 2001). This data was obtained in a dissolution study when a pH gradient-enzymatic method was used, where dissolution was followed until complete release of the drug (Tozaki et al., 2002; Mladenovska et al., in press).

5-ASA concentration was assayed by an UV/VIS spectrophotometer at 300 nm in the clear supernatant solutions after centrifugation and separation of the microparticles (UV/VIS spectrometer, Perkin–Elmer Lambda 16, USA). Blank runs showed absence of significant interference with the spectrophotometric measurement.

2.4.4. Calcium content in the microparticles

Microparticles (250 mg) were dissolved in 10 ml concentrated nitric acid by boiling. The calcium content was determined by atomic absorption spectroscopy (240 nm, ThermoSolaar S4, Great Britain).

2.4.5. Morphological analysis

Shape and surface characteristics of empty and 5-ASA loaded chitosan–Ca–alginate microparticles were studied by scanning electron microscopy (SEM; JEOL JSM—6301F Scanning Microscope, Tokyo, Japan). The samples were mounted on metal grids using double-sided tape and than analysed at 30 kV acceleration voltages after gold coating (thickness 2 nm) under vacuum (BAL-TEC MED 020 Coating system, Austria).

Optical microscopy (technique of phase contrast; Nikon E-800, Japan) was used to observe the morphology of the particles after suspending in isotonic solution containing Tween 80, 1% (w/w).

2.4.6. Polymers distribution within the microparticles

The localization of both the polymers in the microparticles with regard to their respective distribution within the particle and/or wall material was determined using confocal laser scanning microscopy (CLSM). The labeling protocol was based on a method published by Huang et al. (2002) and Schreiber and Haimovich (1983) with minor modifications in respect to the polymer and fluorescent dye ratio, polymer and fluorescent dye solvents and labeling time. FITC and RBITC were chosen to allow simultaneous detection in different channels. The concentrations of the fluorescent markers were adjusted according to their stability in the laser light.

In short, to the solution of chitosan in 1% (w/w) acetic acid the solution of FITC in methanol was added. After 1 h reaction in the dark at 40 °C, the FITC-labeled chitosan was precipitated in 0.2 M NaOH and separated by centrifugation at 4000 rpm for 15 min (Jouan B 3-11, France). The unreacted FITC was removed before freeze-drying by dialysis (dialysis

tube Spectra/Por® 4, MWCO 12,000–14,000) against distilled water under darkness until no diffusion of the free marker was observed.

RBITC was used for labeling of alginate. Aqueous polymer solution was adjusted to pH 8.0 by sodium hydroxide solution (1 M). RBITC dissolved in DMSO at a concentration of 1 mg/ml was added to the polymer solution and stirred for 1 h at 40 °C. The reaction was stopped by adding ethanolamine; free RBITC was removed by dialysis under the same conditions as for chitosan.

The degree of substitution was determined by dissolving the labeled polymers in acetic acid for chitosan–FITC and in water for alginate–RBITC, respectively, and diluting with phosphate buffer, pH 8.0, to measure the concentrations. UV–vis spectroscopy was used to determine the degree of substitution (490 nm for chitosan–FITC, 560 nm for alginate-RBITC, UV/VIS Spectrophotometer 1601 Shimadzu, Japan). The degree of substitution was found to be 0.7 for chitosan, i.e. 7 molecules per 10 sugar units, while for alginate, it was 0.01, which means 1 molecule per 100 sugar units.

The simultaneous visualisation of the two, FITC and RBITC, labeled polymers into the particles has been proven by a separate visualisation of the particles prepared of one labeled polymer. Olympus light microscope Fluoview FU 300 Laser Scanning Confocal Imaging System (Paris, France) equipped with an argon ion laser (EM 488 nm) and helium–neon ion laser (EM 543 nm) was used to investigate the structure and the morphology of the microparticles. All confocal fluorescence pictures were taken with a 60× objective (oil immersion). The laser was adjusted in the green/red fluorescence mode which yielded two excitation/emission wavelengths at 488/520 nm for FITC and at 543/572 nm for RBITC, respectively. Green and red images were obtained from two separated channels and a third picture from the superposition of the green and red picture. For imaging the different sections of the microparticles, two parallel *x*, *y*-planes were scanned at a distance of 0.1 or 0.2 μm by using the Z-series mode.

2.4.7. Evaluation of the polymers–drug interaction using Fourier transform infra-red measurements (FTIR)

FITR measurements were taken at an ambient temperature using Bruker Vector 22 (Germany). About 2 mg of the samples were ground thoroughly with KBr and pellets were formed under a hydraulic pressure of 600 kg/cm^2. The characteristic absorption bands for the polymers and the drug, respectively, were determined in samples of pure substances; their physical mixtures, blank alginate– and chitosan–Ca–alginate microparticles and 5-ASA loaded alginate– and chitosan–Ca–alginate microparticles.

Physical mixtures of 5-ASA and alginate (1:6 ratio, 5-ASA content 14.28%) and 5-ASA, alginate and chitosan (1:6:2.7 ratio, 5-ASA content 10.28%) were ground into a fine powder and 2 mg of each sample was mixed with dry KBr and then compressed into pellets as described before. The same samples of physical mixtures were used for DSC and X-ray diffraction studies (described below).

2.4.8. Evaluation of the polymers–drug interaction and physical state of 5-ASA in the microparticles using differential scanning calorimetry (DSC) and X-ray diffraction studies

DSC studies were performed using a DSC Mettler Toledo model 30TC 15 (Mettler, Zurich, Switzerland). The samples (2–5 mg) were scanned in sealed aluminium pans under nitrogen atmosphere. DCS thermograms were scanned in the first heating run at a constant rate of 10 °C/min and a temperature range of 0–325 °C. DSC thermograms of pure substances, their physical mixtures, blank- and drug-loaded microparticles were recorded.

X-ray diffraction pattern for 5-ASA alone was recorded at 20 °C with Bragg–Brentano geometry on a Philips PW1830 model (Holland), Cu Kα1 radiation, λ = 1.5406 Å, slit width of 1°, in the 2θ range from 5° to 45°, in steps of 0.01° at 1 s per step, while diffraction patterns for the physical mixtures of polymers and 5-ASA and 5-ASA loaded microparticles were recorded in steps of 0.05° at 15 s per step.

3. Results and discussion

3.1. Stability tests of 5-ASA in solutions and in microparticles

The spray-drying method followed by ionotropic gelation/polyelectrolyte complexation here described appeared to be a suitable and relatively simple technique to prepare 5-ASA loaded chitosan–Ca–alginate microparticles. Formulation of the primary dispersion of alginate and 5-ASA for spray-drying in respect to its pH value was of crucial importance considering not only the affinity of the drug for the polymer, but also the 5-ASA solubility and stability in different pH solutions. Literature data (Zerrouk et al., 1998) indicate that 5-ASA solubility increases with increasing pH (alginate's one as well), but it can be rapidly degraded especially in an alkaline medium. It is also a light, temperature and oxygen sensitive drug (Palsmeier, 1992). In this respect, in the actual stability studies the formulation, preparation and 5-ASA release conditions were considered.

In the UV absorption spectra of 5-ASA alone and with alginate in PBS (λ_{max} 328 nm, pH 7.0) and in HPLC water with pH 7.0 (adjusted with 0.2 M NaOH) no additional peaks were observed during 3 days when solutions maintained at room temperature. However, the 4th day although the samples were protected from the light, it was found that exposure to the neutral pH medium resulted in a change in color of the solution, which was considered as a qualitative indication for the degree of 5-ASA degradation. Furthermore, an additional peak in the spectrum at 420 nm was observed (figure not presented), which can be explained by formation of tetrameric species of 5-ASA (Jensen et al., 1992). In addition, the stability tests of 5-ASA in aqueous mediums with pH values between 7.2 and 8.2 showed relatively fast and intensive coloration making them unsuitable for microparticle preparation despite higher 5-ASA solubility. Considering that, a compromise between 5-ASA stability and solubility was made and an aqueous solution of 5-ASA and alginate with pH 7.0 was chosen as an optimal medium for spray-drying. The intensive coloration obtained the 4th day under

Table 1
^{1}H NMR spectra of 5-ASA in aqueous mediums with different pH values

Assignment	Proton signal δ (ppm) (J, Hz)			
5-ASA (pH)	3.0[a]	5.0[b]	7.0[c]	7.4[d]
H-6, H-2′	7.88 (3999.58)	7.55 (3772.43)	7.20 (3597.57)	7.19 (3592.85)
H-4, H-6′	7.36 (3645.43)	7.16 (3587.46)	6.89 (3443.74)	6.88 (3440.81)
H-3, H-5′	6.99 (3.6031)	6.85 (3425.57)	6.72 (3359.68)	6.71 (3357.19)

[a] Solution 5-ASA in 1% (w/w) acetic acid (0.5%, w/w).
[b] Aqueous solution of 5-ASA (0.5%, w/w).
[c] Aqueous solution of 5-ASA (0.5%, w/w), pH 7.0 adjusted with 0.2 M NaOH.
[d] Solution of 5-ASA in PBS (0.5%, w/w).

these conditions underlined the necessity of considering all the precaution measures in order to prevent 5-ASA degradation during preparation of 5-ASA-alginate solution for spray-drying. Besides light protection, they included freshly prepared solutions immediately before spray-drying and use of nitrogen to prevent oxidative self-coupling of 5-ASA moieties.

When working in such conditions, no additional degradation products were detected, which was confirmed by ^{1}H NMR spectra of 5-ASA also. No differences in the ^{1}H NMR spectra of 5-ASA in mediums with different pH values were observed during 4 days. The shift of the proton signal (δ, ppm) in the different pH solutions originated from the ionisation processes of 5-ASA only (Table 1, Fig. 1).

In order to evaluate the 5-ASA stability during spray-drying (under defined pressure and temperature) and gelation/polyelectrolyte complexation procedure, ^{1}H NMR spectra of 5-ASA were registered after solubilization of 5-ASA loaded alginate microparticles in water and PBS (pH 7.0–7.4) and after 5-ASA release from chitosan–Ca–alginate microparticles

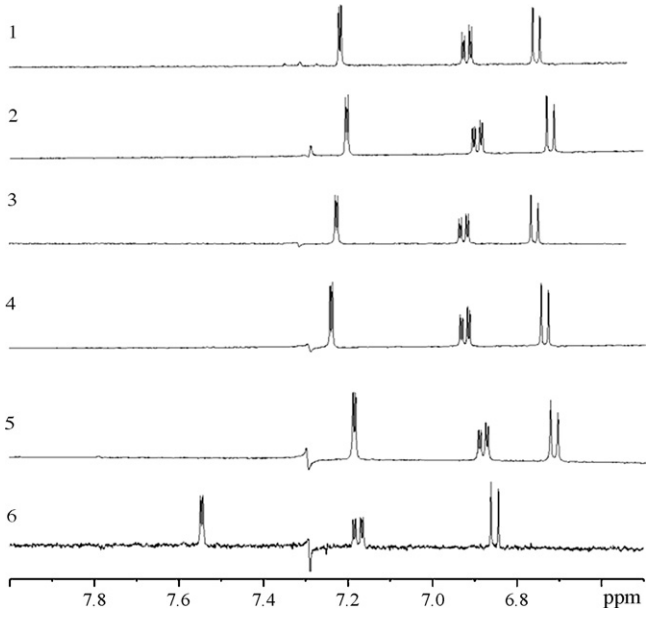

Fig. 1. ^{1}H NMR (500 MHz, δ (ppm)) spectra of 5-ASA in different pH mediums: (1) aqueous solution of 5-ASA (pH 7.0 adjusted with 0.2 M NaOH); (2) aqueous solution of 5-ASA (adjusted pH 7.0) 4 days of preparation; (3) aqueous solution of 5-ASA and alginate (1:3 w/w), adjusted pH 7.0; (4) 5-ASA released from alginate– and chitosan–Ca–alginate microparticles in PBS pH 7.0; (5) 5-ASA released from alginate– and chitosan–Ca–alginate microparticles in PBS pH 7.4; (6) aqueous solution of 5-ASA (pH 5.0).

in these mediums. This includes also the influence of freeze-drying process on 5-ASA stability. ^{1}H NMR spectra of 5-ASA in all samples corresponded to the ^{1}H NMR spectra of 5-ASA in the mediums with adequate pH (Fig. 1).

3.2. Particle size analysis, zeta potential and 5-ASA content

The formulation and preparation process resulted in production of negatively charged particles with a d_{vs} of 6.2 ± 2.2 μm and calcium content of 3.6 ± 0.2%. Zeta potentials of blank Ca–alginate–, blank chitosan–Ca–alginate- and 5-ASA loaded chitosan–Ca–alginate microparticles were -46.1 ± 1.7 mV, -32.1 ± 1.2 mV and -30.7 ± 1.8 mV, respectively. No remarkable difference was found in particle size and distribution between blank- and drug-loaded microparticles, indicating that the loading of 5-ASA in the microparticles substantially did not influence their size. Real drug content was 8.86 ± 0.69%, which is satisfactory 5-ASA content comparing with the theoretical value (14.28%) (Mladenovska et al., in press).

3.3. Morphological analysis

By imaging with SEM, an acceptable spherical morphology was observed, but also flattened, disk-shaped particles. The surface appeared smooth with low porosity and no free drug, i.e. needle-like structures were present. The absence of ideal spherical morphology can be probably attributed to the drying process that causes certain invaginations in the particles (Fig. 2a). The particles tend to agglomerate (Fig. 2b), probably due to the specific localization of the polymers and existence of attractive electrostatic forces. It must be emphasized that the morphology of freeze-dried particles was observed. All blank microparticles were characterized by morphology similar to that of the drug loaded microparticles (Fig. 3).

In addition, the optical microscopy (technique of phase contrast) was used to observe the morphological parameters of the particles after suspending in isotonic solution containing Tween 80, 1% (w/w). Disaggregating of the particles followed by fast swelling was observed. Therefore, invaginations were corrected and the particles obtained spherical shape (image not presented).

3.4. Polymers distribution within the microparticles

CLSM provides a method for three-dimensional reconstruction and image analysis of the microparticles by imaging several

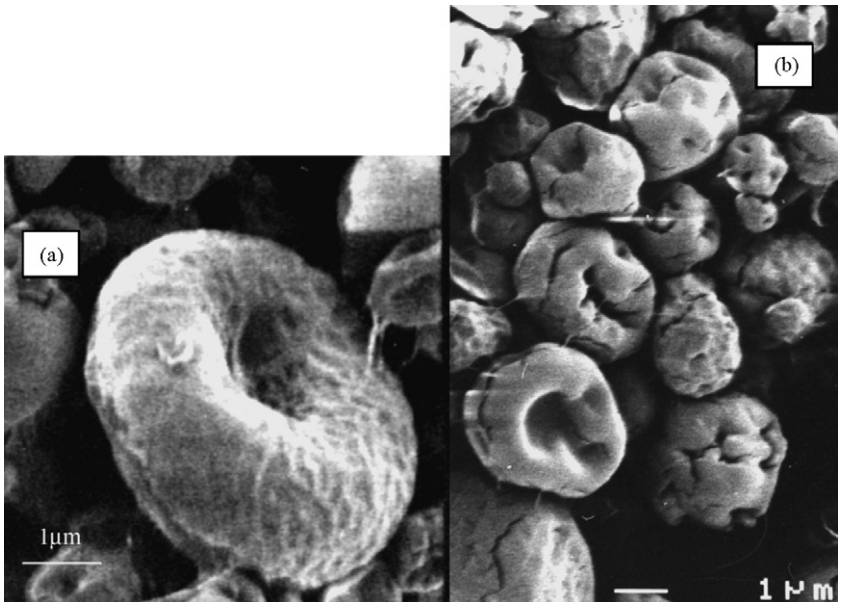

Fig. 2. (a and b) SEM of 5-ASA loaded chitosan–Ca–alginate microparticles.

coplanar sections throughout the object. Moreover, CLSM allows visualization and characterization of structures not only on the surface, but also inside the particles, provided the material is sufficiently transparent and can be fluorescently labeled (Lamprecht et al., 2000a). Thus, the polymers' distribution within the particles was followed by separate fluorescent labeling of each of the polymers. Fluorescently labeled polymers were simultaneously used for preparation of 5-ASA loaded chitosan–Ca–alginate microparticles.

By imaging with CLSM, the FITC-labeled chitosan was localized dominantly in the particle wall with a low quantity homogeneously distributed throughout the particle matrix (Fig. 4a and c). Homogeneous distribution of the alginate throughout the particle wall and matrix was observed with certain zones of heterogeneity marked by the fluorescence signal, which points to the concentrated alginate at the interface with chitosan due to the electrostatic attraction of oppositely charged polymers (Fig. 4b and c). The observation was confirmed by the separate visualization of the particles prepared with one polymer labeled only.

According to the literature data (Lamprecht et al., 2000b), polymers' properties do not change during spray-drying and they do preserve polyionic character and surface activity. This explains the observed homogeneous distribution in the particle wall and matrix with heterogeneity zones.

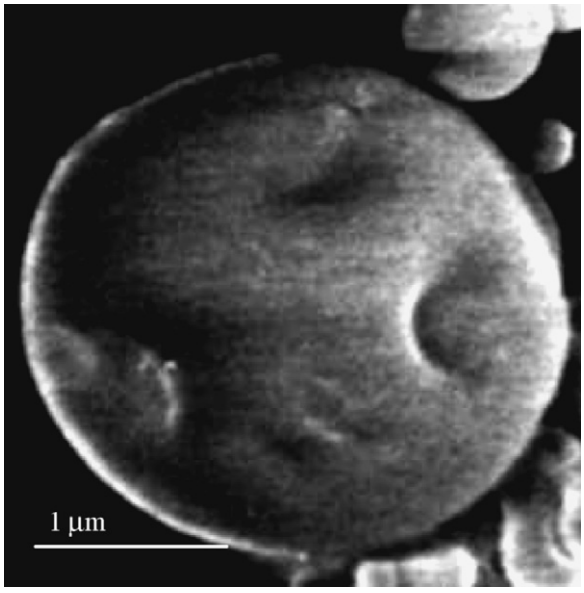

Fig. 3. SEM of blank chitosan–Ca–alginate microparticles.

3.5. Polymers–drug interaction analysis using FTIR spectroscopy

According to the characteristic spectra of the polymers and 5-ASA separately, in physical mixtures and in the microparticles, an attempt was made to detect the eventual existence and type of interactions between the polymers and the drug. The characteristic alginate spectra have been already described (Wang and He, 2002; Soares et al., 2004) and they correspond to: –CH (295 cm^{-1}), –COO (1620 cm^{-1}), –CH (1430 cm^{-1}) and C–O–C (1040 cm^{-1}), while the band at 3440 cm^{-1} belongs to the stretching vibrations of the hydroxyl groups bonded via hydrogen bonds (Figs. 5-2 and 6-2).

Chitosan spectra has been also recorded (Mi et al., 2002) and they are characterized by a broad absorption around 1660 cm^{-1} (Amide I, C–O stretching mode conjugated with –N–H deformation mode) and 1600 cm^{-1} (δNH_2 bend of non-acetylated NH2 groups). Characteristic for its saccharide structure are absorption bands at 1154 cm^{-1} (asymmetric stretching vibration of the C–O–C bridge), 1083 cm^{-1} and 1038 cm^{-1} (skeletal vibration involving C–O stretching) (Fig. 5-3).

Considering 5-ASA, the characteristic IR bands at 3445 cm^{-1} owing to the mutual overlapping of νNH and νOH stretch-

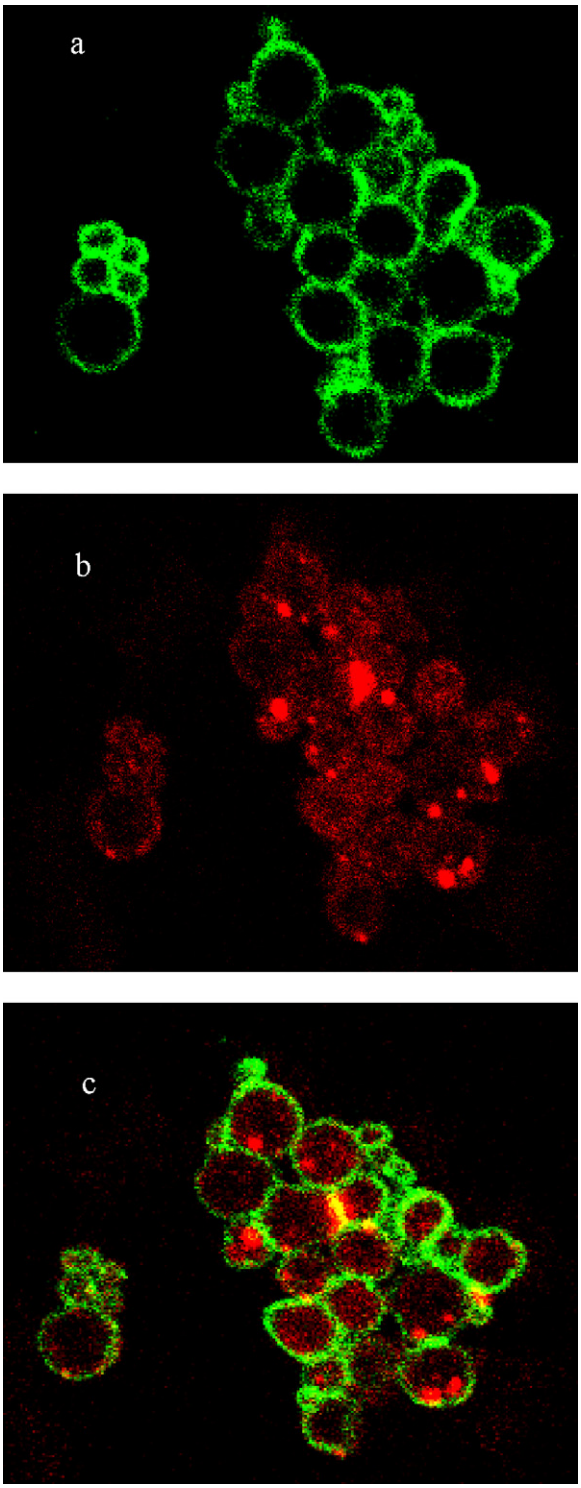

Fig. 4. CLSM of 5-ASA loaded chitosan–Ca–alginate microparticles; FITC labeled chitosan (a); RBITC labeled alginate (b); image obtained by superposition (c).

ing were observed. The peak at 1650 cm^{-1} corresponds to the C=O stretch, NH (bend) is assigned by the peak at 1620 cm^{-1}, while C–N stretch with the peak at 1356 cm^{-1}. The bands in a range of 2000–3000 cm^{-1} correspond to the stretching vibrations of the hydrogen bonds in the 5-ASA molecule (Figs. 5-1 and 6-1).

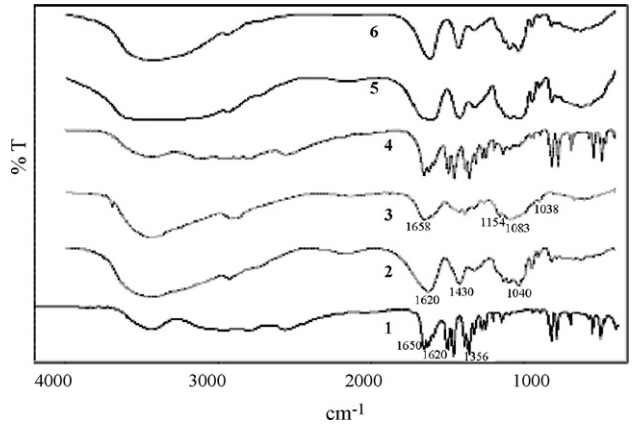

Fig. 5. FTIR spectra of chitosan, alginate, 5-ASA and corresponding physical mixtures and microparticles: (1) 5-ASA; (2) sodium alginate; (3) chitosan; (4) physical mixture of 5-ASA, sodium alginate and chitosan; (5) blank chitosan–Ca–alginate microparticles; (6) 5-ASA loaded chitosan–Ca–alginate microparticles.

Electrostatic interactions between carboxyl groups of alginate and amino groups of chitosan and existence of interactive coulomb forces have been already described (Wang et al., 2001; Ribeiro et al., 2005). In the FTIR spectra of the physical mixtures of alginate and 5-ASA (Fig. 6-3) and chitosan, alginate and 5-ASA (Fig. 5-4), the characteristic 5-ASA peaks can be observed, suggesting absence of interactions between the drug and the polymers.

In order to define the interactions between the drug and the polymers in the particles, samples of 5-ASA loaded alginate– and chitosan–Ca–alginate microparticles were investigated. FTIR spectra of 5-ASA incorporated in alginate microparticles (Fig. 6-5) were compared with those of blank alginate microparticles (Fig. 6-4). The presence of characteristic IR bands of 5-ASA at 1494, 1454, 1356, 774, 538 and 485 cm^{-1} in the spectra of 5-ASA loaded alginate microparticles suggests absence of high affinity interaction between alginate and 5-ASA in the particles. However, the characteristic peaks of 5-ASA could not be observed in the chitosan–Ca–alginate microparticles (Fig. 5-6). The content of 5-ASA in the chitosan–Ca–alginate

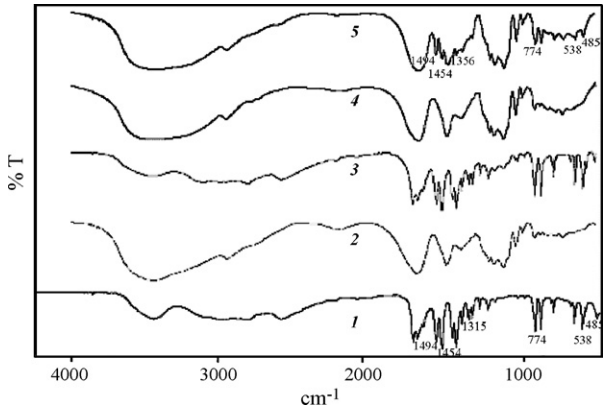

Fig. 6. FTIR spectra of alginate, 5-ASA and corresponding physical mixtures and microparticles: (1) 5-ASA; (2) sodium alginate; (3) physical mixture of 5-ASA and sodium alginate; (4) blank alginate microparticles; (5) 5-ASA loaded alginate microparticles.

microparticles (8.86%) did not differ significantly from the content of 5-ASA in the physical mixture of 5-ASA, alginate and chitosan (10.28%; Fig. 5-4). Considering that, the IR spectra of 5-ASA loaded chitosan–Ca–alginate microparticles may be explained by existence of interactions (ionic, hydrogen and/or van der Waals' forces) between 5-ASA, alginate and chitosan occurring during complexation/gelation process, which results in drug embedding in the particles. Embedding in the polymers' matrix may increase stretching barrier for 5-ASA intra-molecular bonds, so the characteristic 5-ASA peaks could not be identified. In addition, ^{1}H NMR, X-ray and DSC studies confirmed 5-ASA stability and embedding in the particles at a molecular level.

3.6. Polymers–drug interaction analyses using DSC and X-ray diffractometry

In the calorimetric studies, the thermogram of alginate scanned in the first heating run is characterized by a sharp endothermic peak around 155 °C and a broad exothermic peak at 240 °C (Figs. 7-2 and 8-2). The first peak attributes to a dehydration process followed by decomposition of the polymer, while the second one denotes the temperature of alginate degradation (Soares et al., 2004). Additionally, in the thermogram of alginate scanned in the second heating run, the change of inclination of the baseline at 110 °C was observed, which corresponded to the Tg for alginate. The exothermic peak in the thermogram of alginate in a form of a sharp exotherm around 250 °C exists in the thermograms of the physical mixtures of alginate and 5-ASA (Fig. 7-3) and alginate, chitosan and 5-ASA (Fig. 8-4) pointing to the degradation of alginate. The same was observed in the thermograms of alginate– (Fig. 7-4), Ca–alginate– (Fig. 7-6) and 5-ASA loaded alginate– (Fig. 7-5) and Ca–alginate microparticles (Fig. 7-7).

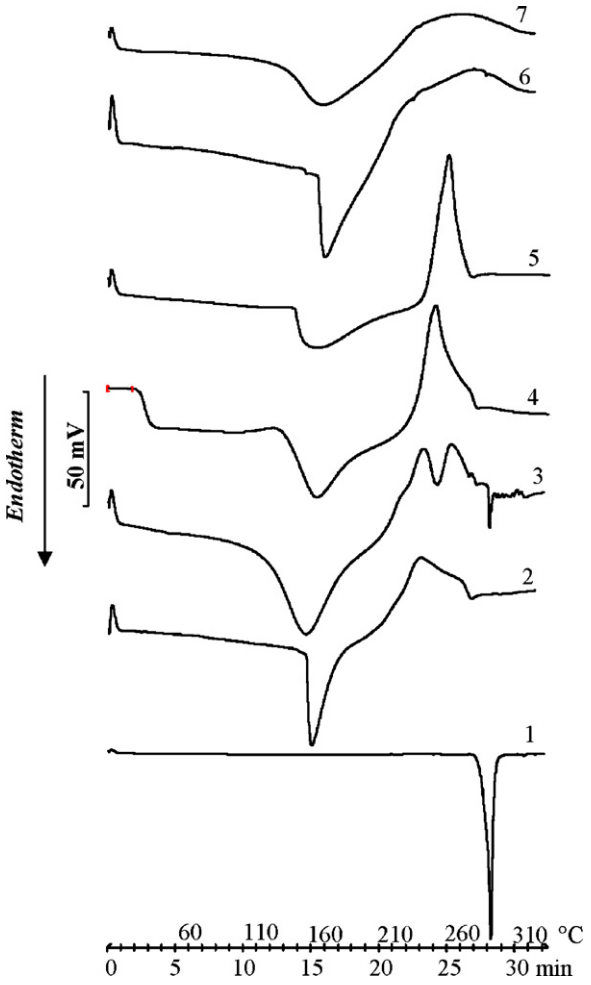

Fig. 7. DSC of: (1) 5-ASA; (2) sodium alginate; (3) physical mixture of 5-ASA and sodium alginate; (4) blank alginate microparticles; (5) 5-ASA loaded alginate microparticles; (6) blank Ca–alginate microparticles; (7) 5-ASA loaded Ca–alginate microparticles.

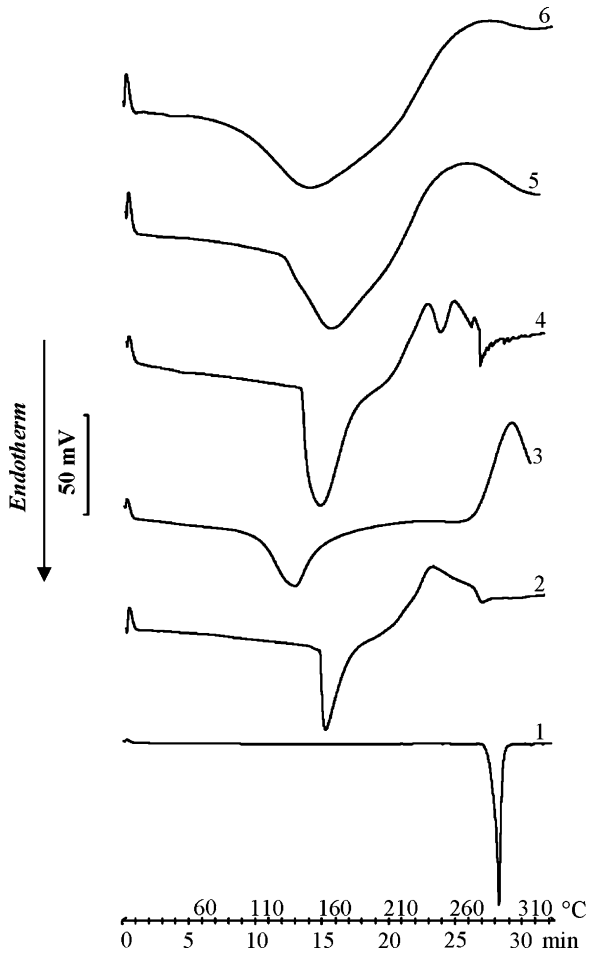

Fig. 8. DSC of: (1) 5-ASA; (2) sodium alginate; (3) chitosan; (4) physical mixture of 5-ASA, sodium alginate and chitosan; (5) blank chitosan–Ca–alginate microparticles; (6) 5-ASA loaded chitosan–Ca–alginate microparticles.

Exchanging sodium ions with calcium ions in the sodium alginate resulted in significant broadening of this exothermic peak at a range of 210–310 °C, which can be explained by the "egg-box" conformation in the Ca–alginate microparticles (Fig. 7-6). The calcium–alginate interaction was confirmed by a sharp endothermic peak around 170 °C also (Fernandez-Hervas et al., 1998; Gonzales-Rodriguez et al., 2002) and by increase in the glass transition temperature for 20 °C, which was detected in the thermogram of Ca–alginate microparticles scanned in the second heating run (thermogram not presented). In addition,

calcium chloride showed two endothermic peaks at a range of 180–220 °C (thermogram not presented), which cannot be seen in the thermogram of Ca–alginate microparticles with and without 5-ASA.

The thermogram of chitosan scanned in the first heating run showed a broad endotherm around 130 °C and a sharp exotherm at 310 °C (Fig. 8-3). The change of inclination of the baseline at 203 °C in the thermogram scanned in the second heating run denotes the Tg. As in alginate, the first peak in the thermogram scanned in the first heating run corresponds to the water evaporation, while the second peak corresponds to the chitosan degradation (Dong et al., 2004). This exotherm exists in the mixture with alginate and 5-ASA also (Fig. 8-4). In the thermogram of blank chitosan–Ca–alginate microparticles, it is transformed in a significantly broad band at a range of 210–320 °C (Fig. 8-5). This exotherm and the endotherms around 130 °C in the thermogram of the physical mixture of chitosan and alginate (thermogram not presented) and around 160 °C in the thermogram of the blank chitosan–Ca–alginate microparticles (Fig. 8-5) denote the polyelectrolyte interaction between the chitosan and alginate.

The thermogram of 5-ASA is characterized by a sharp endothermic peak at 276 °C (Figs. 7-1 and 8-1), which corresponds to its melting point. In the physical mixtures contained of 5-ASA and chitosan (thermogram not presented), 5-ASA and alginate (Fig. 7-3) and 5-ASA, chitosan and alginate (Fig. 8-4), this sharp endothermic peak existed in a range of 260–280 °C and disappeared in the thermograms of 5-ASA loaded alginate- (Fig. 7-5) and Ca–alginate-microparticles (Fig. 7-7). Considering that the thermograms of the blank alginate– and Ca–alginate microparticles do not differ from those of 5-ASA loaded microparticles one can conclude that the embedding of 5-ASA in the alginate matrix in the phase of spray drying is in a molecular form.

The thermogram of the empty chitosan–Ca–alginate microparticles (Fig. 8-5) was not different from that of 5-ASA loaded chitosan–Ca–alginate microparticles (Fig. 8-6). This suggests that 5-ASA does not re-crystallize in the particles in the phase of gelation/polyelectrolyte complexation and confirms the above-mentioned conclusion.

These results were confirmed by X-ray diffraction studies also. The diffractogram of 5-ASA alone had peaks at 15.2° and 16.5°. The diffractogram remained unchanged when 5-ASA in physical mixture with chitosan and alginate (Fig. 9-4). The intensity at 15° in the physical mixture did not correlate with the quantity of 5-ASA. Also, the peak in the physical mixture was broader (half of the height), which generally reveals smaller crystals that were probably formed in the mixing phase. The change may be attributed also to the longer accumulation time (factor 15) and the presence of amorphous material that diffuses more or less from each angle without Bragg's peak.

Fast scanning of 5-ASA alone in a greater range, 2–60°, resulted in one peak more around 8°, which was absent in the other samples, except in the physical mixture of 5-ASA, chitosan and alginate (Fig. 10).

Both the peaks at 15.2° and 16.5° disappeared in the diffractogram of 5-ASA loaded chitosan–Ca–alginate microparticles (Fig. 9-3). Attempt was made to accumulate long enough by concentrating in a region of 14–18° in order to obtain a signal with 5-ASA loaded chitosan–Ca–alginate microparticles. No peak was obtained, which undoubtly confirms that 5-ASA does not re-crystallize in the particles, but it is embedded in the polymeric matrix at a molecular level.

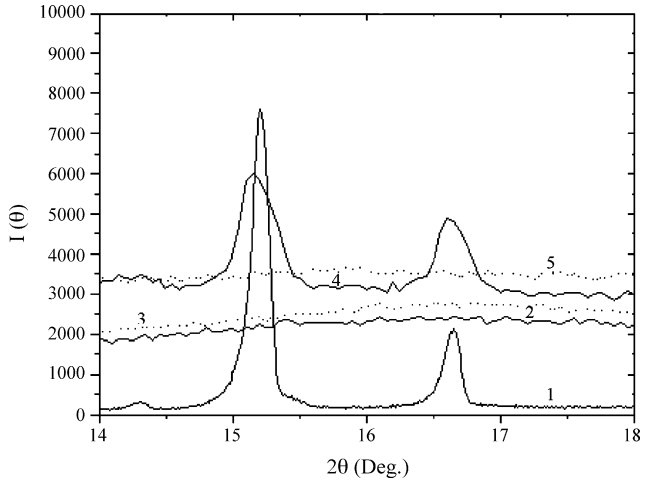

Fig. 9. X-ray diffractograms of: (1) 5-ASA; (2) blank chitosan–Ca–alginate microparticles; (3) 5-ASA loaded chitosan–Ca–alginate microparticles; (4) physical mixture of 5-ASA, chitosan and sodium alginate; (5) physical mixture of chitosan and sodium alginate.

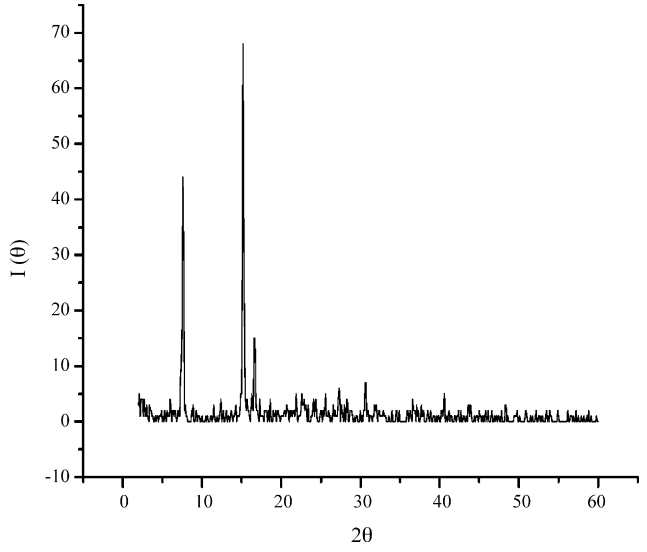

Fig. 10. X-ray diffractograms of 5-ASA (alone and in a mixture with chitosan and sodium alginate).

4. Conclusions

In a conclusion, spray-drying method followed by ionotropic gelation/polyelectrolyte complexation resulted in preparation of negatively charged 5-ASA loaded chitosan–Ca–alginate microparticles with a size less than 10 μm. Acceptable spherical morphology was observed, but also flattened, disk-shaped particles with a smooth surface and low porosity. The chitosan was dominantly localized in the particle wall, while for alginate,

a homogeneous distribution throughout the particles was observed. ^{1}H NMR and UV–vis spectra of 5-ASA have shown no degradation when working in adequate conditions, such as light protection, freshly prepared solutions and use of nitrogen to prevent the oxidative self-coupling of 5-ASA moieties. In the FTIR spectra of 5-ASA loaded Ca–alginate microparticles, the characteristic peaks of 5-ASA were not altered indicating no covalent interaction between the drug and the polymer. The thermograms of 5-ASA loaded Ca–alginate– and chitosan–Ca–alginate microparticles were almost identical to those of empty particles, indicating molecularly dispersed drug within the particles during the spray-drying. The same was confirmed by X-ray studies. Considering physicochemical characterization, one can conclude that 5-ASA loaded microparticles have potential for intensive mucoadhesion and controlled colon-specific delivery, which was also confirmed by dissolution and biodistribution studies of ^{131}I-labeled 5-ASA after peroral administration of these microparticles to rats in which TNBS colitis was induced (Mladenovska et al., in press).

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