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Characterization of azacytidine/poly(L-lactic) acid particles prepared by supercritical antisolvent precipitation

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ABSTRACT

A controlled azacytidine release system based on drug encapsulation with a polymer material has been prepared and characterized. The drug systems were prepared by precipitation from solutions, using supercritical CO_2 antisolvent technique operating in a semi-continuous mode. Azacytidine was dissolved in dimethylsulfoxide and poly-lactic acid in methylene chloride. The two solutions were mixed before being sprayed into the supercritical reactor. Experimental conditions were 40 °C for temperature, 11 MPa for pressure, and a CO_2 flow rate of 30 ml min^{-1}. The precipitated drug–polymer particles were further characterized to determine the percentage of encapsulated drug and establish the delivery kinetics under various release conditions. A sustained delivery of the drug over a period of various hours was obtained. Besides, an improved stability of the coated drug with respect to the pure azacytidine was found, thus proving the suitability of this approach for dealing with unstable compounds.

1. Introduction

In the last 20 years, new technologies focused on encapsulating and immobilizing of chemical compounds in polymeric structures have been developed [1–6]. Regarding pharmaceuticals, the polymer may protect the drug from the physiological environment thus enhancing in vivo stability by avoiding degradation and premature metabolism. As a result, encapsulation of drugs and impregnation of polymers are highly attractive for the elaboration of controlled delivery systems.

Controlled drug release systems are increasingly used as a way of enhancing the effectiveness of the therapeutic treatments by maintaining plasmatic concentrations within the desired levels for long periods of time. The incorporation of specific additives in the system may be exploited for guiding the active agent towards specific locations in the body or for modulating the delivery kinetics. In addition, special polymers such as those pH-sensitive may provide a selective drug delivery in the desired regions of the gastrointestinal tract.

The preparation of drug delivery systems by supercritical CO_2 ($SCCO_2$) technologies is receiving increasing attention as a way of avoiding or reducing the use of organic solvents [7–12]. Among other possibilities, supercritical antisolvent processes (SAS) have proved to be an efficient strategy for dealing with polar drugs [13–15]. SAS produces solid particles from a mother liquor upon addition of a fluid partially or totally miscible with the organic solvent of the mother liquor and that is a weak-solvent for the solute. The $SCCO_2$ addition decreases the solvation ability of the organic solvent and causes the solute precipitation. Temperature and pressure are among the most relevant variables affecting the process performances.

There is a large variety of polymers commercially available for implementing delivery systems. Poly(esters) are among the most studied and best characterized of the biodegradable polymers [16]. They include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), their copolymers poly(lactic acid-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL). The polymer(s) is(are) chosen depending on the drug features and the desired properties of the resulting products, including release mechanisms, biocompatibility and physico-chemical characteristics. An interesting possibility relies on using blends in which the resulting coating properties can effectively be altered by varying the polymer:polymer blend ratio [17].

In this study, 5-azacytidine has been chosen as a model of labile drug for preparing sustained delivery systems. Azacytidine is a chemical analog of cytidine acting as an inhibitor of the DNA methylation in human cells. Azacytidine is receiving increasing attention as a cytostatic agent for the treatment of myelodysplastic syndrome, a preleukemic bone marrow disorder [18–21]. The poor stability of this compound [22–25] may result in a significant drawback

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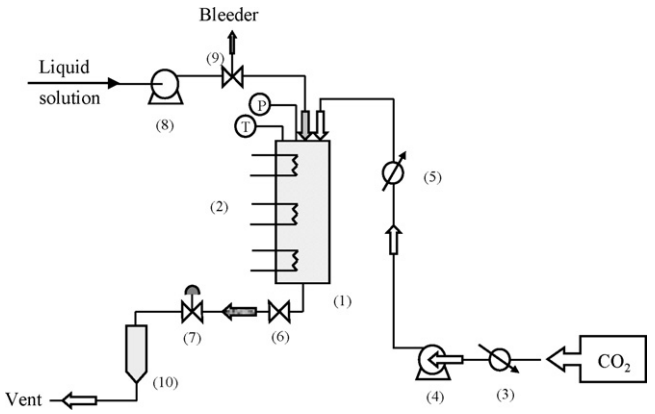

Fig. 1. Scheme of the SAS system. (1) High-pressure vessel, (2) heating jackets (resistances), (3) cooler, (4) reciprocating pump, (5) pressure measurement, (6) valve, (7) metering valve, (8) dual-piston minipump, (9) valve and (10) cyclone.

affecting the therapeutic activity. Hence, the development of strategies for enhancing the stability and bioavailability through devices of controlled delivery is welcome. Here, drug entrapment in a polymeric material by means of SAS technology has been proposed for preparing controlled release systems. Liquid chromatography, scanning electron microscopy and differential scanning calorimetry analysis have been utilized to evaluate the characteristics of the resulting samples.

2. Experimental

2.1. Reagents and solutions

Azacytidine was purchased from Sigma–Aldrich (St. Louis, MO, USA). Poly-lactic acid (L-PLA) was in the shape of pellets (L-PLA from Biovalley) and had an average molecular weight of ~100 kDa. Carbon dioxide (99.95%, w/w) was supplied by Air Liquid. Methylene chloride (DCM) and dimethyl sulfoxide (DMSO) were from VWR. Chemicals utilized for characterization techniques were from Merck: 37% (w/w) hydrochloric acid solution, sodium hydroxide, tris(hydroxymethyl)aminomethane (TRIS), methanol, ammonium acetate and acetic acid. Ultrapure water (Millipore, Milford, MA, USA) was used for the preparation of all solutions. Fresh 1×10^{-4} mol l^{-1} 5-azacytidine stock solutions were daily prepared in water and stored at 4 °C.

2.2. Antisolvent equipment and process

Experiments were carried out in a SAS apparatus operating in a semi-continuous mode (see Fig. 1). The spray chamber consisted of a high-pressure vessel (1, Autoclave Engineers) of 5 cm i.d. and 25 cm long with sapphire windows allowing for visual observation of the precipitation. The vessel temperature was controlled by means of heating jackets (2). At the bottom of the vessel, a membrane filter placed on a stainless steel frit of 2 μm porosity allowed for the collection of precipitated solids. The CO_2 was first cooled (3) and then pumped using a reciprocating pump (4, Lewa EK3); the pressure inside the vessel was controlled downstream (5) with a micrometering valve (7).

The solutions that contained the species to be precipitated were sprayed into the vessel using a dual-piston minipump (8, Milton Roy LDC) and a coaxial spray nozzle with an inner capillary of 500 μm i.d. In a typical experiment, once the vessel attained the desired temperature of 40 °C, the CO_2 was introduced while keeping valve (6) closed until the selected pressure was reached (11 MPa). Then, valve (6) was opened and the system was allowed to equilibrate at a CO_2 flow of 30 ml min^{-1}. Next, the mixture of polymer and drug solution was injected into the vessel through a nozzle by opening valve (9). The simultaneous mass transfer of the $SCCO_2$ and of the liquid solvent from one phase to the other one induced the supersaturation of the solution and consequently induced the precipitation of the species. The newly formed CO_2–solvent mixture was separated downstream through a cyclone (10). Once the desired volume of solution was sprayed, the CO_2 flow was maintained in order to dry the precipitated particles. Finally, the system was depressurized across the metering valve (7) at the experimental temperature, and the precipitated particles were collected on vessel walls and bottom.

2.3. Analytical instruments

A magnetic stirrer IKA® RCT basic equipped with a temperature probe was used for controlling the release conditions. A CyberScan model 2500 potentiometer (precision of ±0.1 mV) with a combined pH electrode ORION 9103SC was used for pH measurements. The chromatographic system consisted of an Agilent 1100 Series instrument equipped with a G1311A quaternary pump, a G1279A degasser, a G1315B diode-array detector furnished with a 13 μl flow cell. A differential scanning calorimeter (DSC-822e/400 Mettler Toledo) was used to measure glass and/or melting temperatures. Thermograms were obtained at a heating rate of 10 °C min^{-1} in the range of 30–250 °C under N_2 purge at 50 ml min^{-1}. A Hitachi H-4100FE scanning electron microscope (SEM) was utilized for morphology studies. Prior analysis, samples were gold sputter-coated with a Jeol JFC 1100 apparatus to render them electrically conductive.

2.4. Chromatographic procedure

Samples were injected into the HPLC systems with a Rheodyne 7725(i) (Rohnert Park, CA, USA) 6-port valve with a 20 μl sample loop. The analytical column was a Gemini C_{18} column (150 mm × 4.6 mm i.d., particle size 4 μm, 110 Å) equipped with a guard column (4 mm × 3 mm i.d.), both from Phenomenex (Torrance, CA, USA). The column temperature was 25 °C. The chromatographic eluent consisted of 20 mM TRIS (pH 9.0) + MeOH (98:2, v/v). Isocratic elution at a constant flow rate of 1 ml min^{-1} was utilized. Chromatograms were recorded at 215 nm.

2.5. Determination of the percentage of entrapment

The total amount of azacytidine and related products in the sample preparation was determined by HPLC. For this purpose, 5 mg of sample were treated with 10 ml of methylene chloride for swelling and dissolving the polymeric support while quickly releasing the drug components to the solution. Subsequently, the solvent was evaporated under nitrogen current and the dry residue was re-dissolved in 10 ml of MilliQ water. 20 μl of the resulting solution were injected into the HPLC system for the quantification of azacytidine species.

The uncoated drug fraction including adsorbed plus free drug contributions (burst effect) was determined chromatographically as the drug amount released in water in less than 30 s. The entrapped drug fraction was calculated from the difference between total and burst drug amounts.

2.6. Drug release monitoring

2.6.1. Batch set-up

The drug delivery process was evaluated via desorption experiments in which the solute was lixiviated using appropriate aqueous media, namely: (i) 10 mM HCl (pH 2) and (ii) 10 mM HCO_3^-/CO_3^{2-}

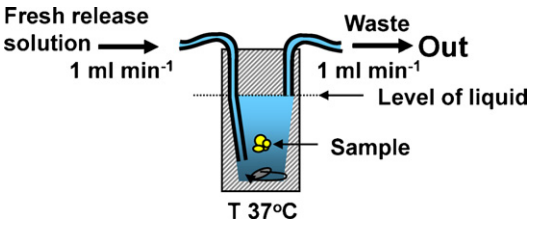

Fig. 2. Scheme of the continuous-flow system for studying drug release under dynamic conditions.

(pH 7.4). A proper amount of sample (~60 mg) was placed in the centre of a vessel containing the desired volume of aqueous solution (50 ml). Stirring rate was fixed at 70 rpm and temperature at 37.0 °C ± 0.1. The kinetic process was monitored chromatographically to obtain the corresponding delivery profiles giving, thus, information about the evolution of azacytidine species in the system. For this purpose, discrete volumes of sample of 500 μl at desired times were collected throughout the process to be further injected into the chromatograph. The principal drawback of this approach was the occurrence of progressive drug decomposition inside the vessel in parallel to the release process. Then, it was impossible to ascertain the characteristics of the freshly released drug as it could not be separated from the concurrent degradation process.

2.6.2. Continuous-flow set-up

Monitoring the release process under dynamic conditions relied on the continuous-flow system described in Fig. 2. The releasing solution (either that of pH 2 or pH 7.4) was continuously pumped through the system at a constant flow rate of 1 ml min^{-1}. The volume of liquid inside the vessel was set to 4 ml. The outlet stream, also flowing at 1 ml min^{-1}, was sent to waste. Small portions of the emerging solution (≈500 μl) were collected in Eppendorfs at desired times over the process to be immediately analyzed by HPLC. In these circumstances, drug from the sample was continuously dissolved in fresh media. At the same time, the decomposition undergone by the drug was almost negligible since the short residence time inside the vessel.

3. Results and discussion

3.1. Azacytidine physico-chemical background

Physico-chemical properties of azacytidine, specially, decomposition kinetics strongly influence on the characteristics of release process. A scheme showing reactions is given in Fig. 3. According to preliminary studies [24], the azacytidine decomposition comprises two steps: first, a rapid and reversible hydrolysis of the heterocyclic nitrogen ring due to a nucleophilic attack on N5 gives N-(formylamidino)-N′-β-D-ribofuranosylurea (RGU-CHO); secondly, the reaction evolves more slowly with an irreversible lost of H_2CO to yield 1-β-D-ribofuranosyl-3-guanylurea (RGU). According to this mechanism, three species are progressively formed over time [25]. Besides, in long-term degradation studies involving several days, another similar molecule (5-aza-2′-deoxycytidine) have shown the formation of additional degradation species from RGU [26,27].

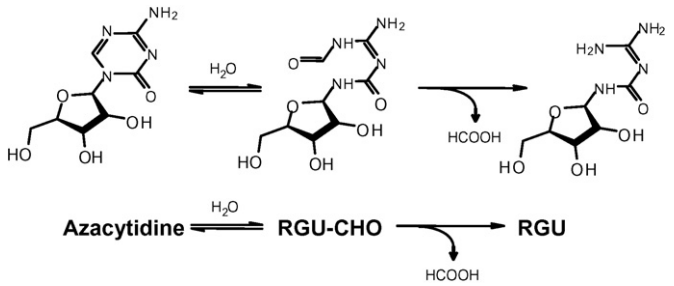

Fig. 3. Scheme of the degradation processes of azacytidine.

3.2. Selection of solvents for SAS process

The choice of appropriate azacytidine and PLA solvents was an important point to keep in mind before starting with SAS experiments. For this purpose, preliminary solubility studies were carried out using various solvents and mixtures. Regarding the polymer, L-PLA was highly soluble in DCM. The drug was found to be highly soluble in water but, unfortunately, this solvent is not recommendable for SAS experiments. First, the immiscibility of water with the organic PLA solvent (DCM) hinders the formation of the solution to be sprayed into de SAS system. Besides, the poor solubility of water in the supercritical fluid hinders the removal of the residual water by supercritical post-processing from the formed particles. Apart from water, azacytidine was also moderately soluble in methanol and DMSO and hardly soluble in acetone, ethanol, 2-propanol, methylene chloride and other organic solvents. From the two potential solvents, methanol was refused due to its toxicity. Hence, the most reasonable choice was DMSO despite being less volatile so that its removal from the final samples may result in a more costly task.

Since the azacytidine and PLA could not be dissolved in the same solvent, a mixture of DMSO (good solvent for azacytidine) and DCM (good solvent for L-PLA) in a volumetric ratio of 50:50 was selected, after verifying that both species were still soluble in the new mixture, at the processed concentrations of 4 mg ml^{-1} for the drug and 15 mg ml^{-1} for the polymer.

3.3. Drug content

The total amount of drug present in the prepared sample was determined by HPLC according to the method described in Section 2. Results indicated that the drug percentage in the sample was 25% (w/w). It should be remarked that the composition of sample produced was similar to the initial azacytidine/PLA ratio (25/75%, w/w). In other words, the precipitation process did not induce any change in the species composition. The chromatographic analysis also revealed a small amount of RGU-CHO corresponding to a 5% of drug (i.e., 1.3% with respect to the total weight). Furthermore, no traces of RGU and other degradation products were observed. The occurrence of RGU-CHO was attributed to the high lability of azacytidine as pointed out elsewhere [24,25]. From the therapeutic point of view, however, the formation of RGU-CHO in the samples was not considered as a serious shortcoming owing to its noticeable cytostatic activity.

Note that the percentage of drug contained in the sample provided scarce information about how it was entrapped or distributed in the product resulting from the SAS experiment. Hence, the drug distribution corresponding to encapsulated and absorbed plus free fraction was characterized as follows. The free and superficial drug fraction was estimated by the so-called burst effect as the amount of drug dissolved in the medium in the first 30 s. This fraction was a 5% of the total drug amount. Hence, it was concluded that the remaining 95% of azacytidine was efficiently entrapped inside the polymeric web. A complementary study was focused on determining the azacytidine/RGU-CHO ratio in this burst fraction. The result was similar to that found for the whole material (i.e., 95:5 as indicated in the previous paragraph). Hence, it suggested that RGU-CHO formation occurred during the SAS process since both the encapsulated and external fractions are affected in a similar way, probably because of DMSO that is a hygroscopic liquid.

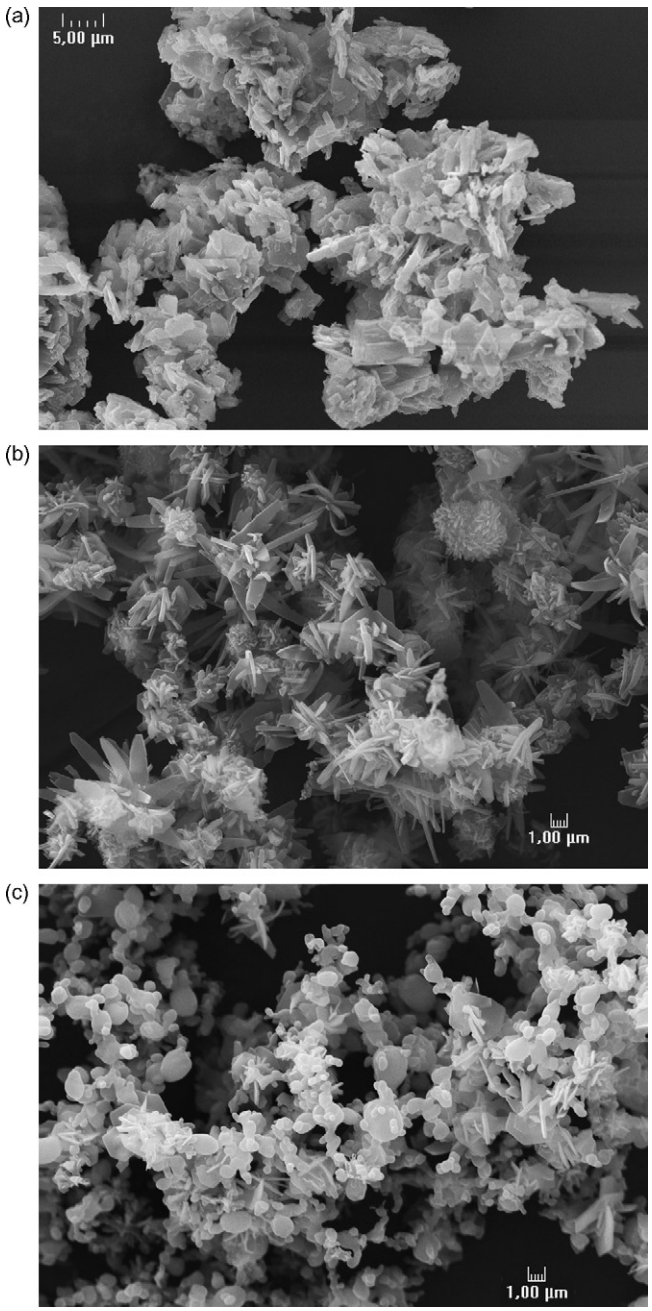

Fig. 4. SEM pictures of unprocessed raw drug (a), precipitated drug (b) and drug–PLA sample (c).

SEM technique was used to document the morphology of produced particles. As an example, Fig. 4 shows the pictures of the pure drug and the drug–polymer particles, both precipitated by SAS, to be compared with the unprocessed raw drug. The unprocessed azacytidine shows a plate-like shape and are highly agglomerated. When processed alone, the drug particles exhibit an elongated shape of dimension below 3 μm, probably due to the capillary device used to spray the solution, and are arranged in star-like structures below 6 μm. In the presence of PLA, however, significant morphologic changes were observed. The deformed balloon-like structures evidenced the coating of the drug by the polymer, since PLA alone precipitated with an almost spherical balloon aspect; some elongated structures are still visible besides the balloon populations. The produced particles showed size roughly below 2 μm. SEM results were consistent with chromatographic results as the vast majority of drug was entrapped in the polymeric structure.

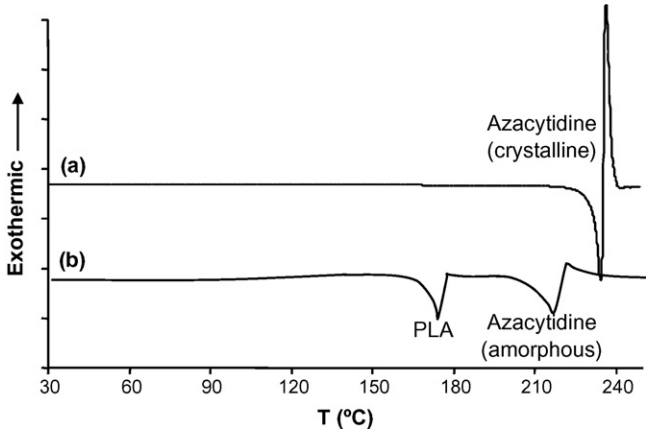

Fig. 5. DSC analysis of raw azacytidine (a) and azacytidine/PLA sample (b).

Differential scanning calorimetry was used to document thermal properties of the CO_2 processed samples. Thermograms of both pure drug and the drug/polymer specimen are compared in Fig. 5. The sharp endotherm peak of Fig. 5a at 235 °C corresponds to the melting temperature of crystalline azacytidine in the raw product. This peak, associated to the drug disappeared in the drug/PLA sample profile so that the drug percentage in crystalline form was negligible; instead, two peaks at 174 and 215 °C were found which correspond to the melting temperatures of polymer and of the amorphous drug residue, respectively. Indeed, L-PLA is a semicrystalline polymer that has a melting temperature of 176 °C. This temperature did not change after being in contact with CO_2, that is an usual behavior for PLA [28]. Again, the absence of the pure drug signal supported that a successful drug entrapment was actually accomplished.

3.4. Drug release monitoring

Drug delivery profiles were assessed chromatographically as described in Section 2. Studies were carried out using the batch and continuous-flow procedures in both acid and basic medium to simulate gastric and intestinal conditions, respectively. The performance of the sustained release and the influence of the encapsulation on the enhancement of the stability were evaluated from a comparison with the behavior of the pure raw drug in analogous experimental circumstances. Especial attention was paid on the discrimination between azacytidine and degradation species. Besides, release profiles were obtained from independent replicate experiments so information about variabilities was also available.

3.4.1. Delivery at pH 2

Fig. 6 summarizes the results from the series of studies at pH 2 which includes the dissolution/stability assays of raw azacytidine as well as the release processes obtained with the batch and continuous-flow procedures.

According to Fig. 6a, the pure drug was dissolved almost instantly in the 10 mM HCl solution. From this initial point, azacytidine concentration continuously decreased over time and, after 7 h, the amount of unaltered drug remaining in the measurement vessel was lower than 8%. In the case of RGU-CHO, an initial slight rise in its concentration was observed up to a maximum of 15% at a time of 15 min. From that time, a progressive decay was produced. In contrast, the kinetic profile of RGU showed a progressive increase over time, thus, resulting in the major species after 7 h of assay.

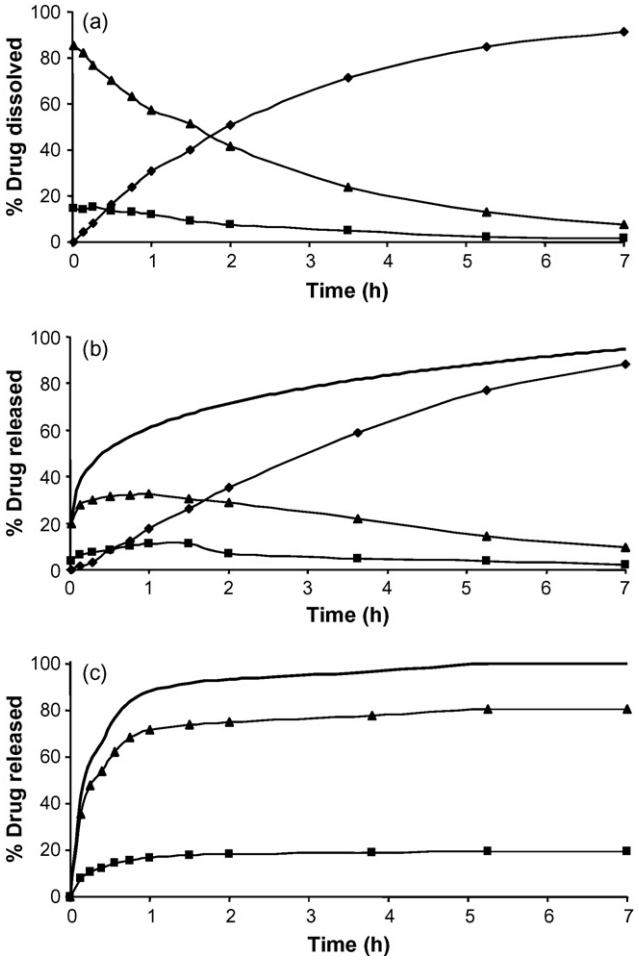

Fig. 6. Drug delivery studies at pH 2. (a) Dissolution/degradation process of the pure drug; (b) release profiles using the batch procedure; (c) release profiles using the continuous-flow procedure. Symbols: triangle = azacytidine; square = RGU-CHO; rhombus = RGU; bold line = total azacytidine species.

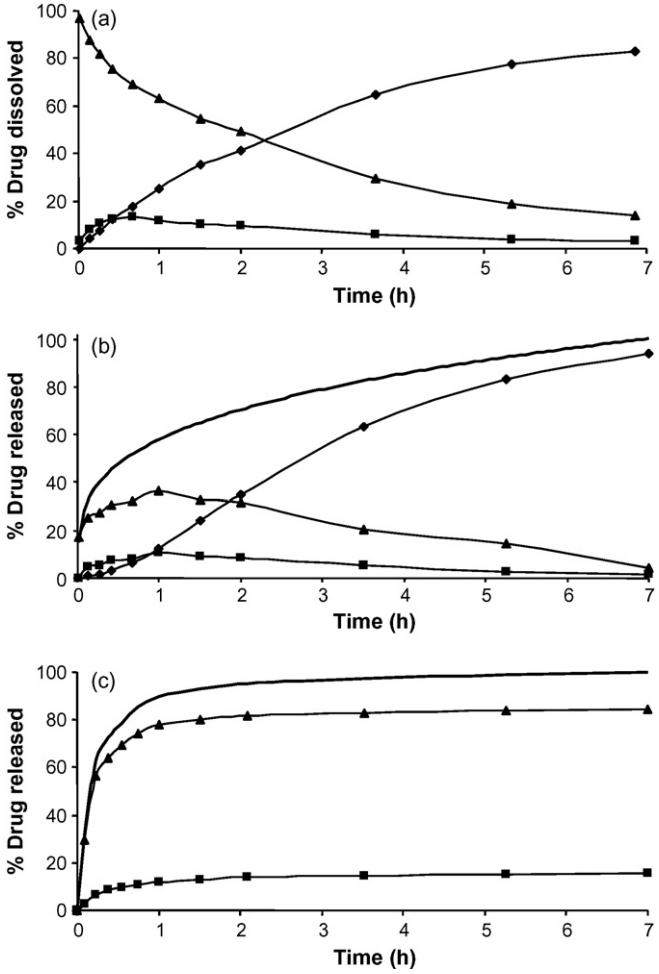

Fig. 7. Drug delivery studies at pH 7.4. (a) Dissolution/degradation process of the pure drug; (b) release profiles using the batch procedure; (c) release profiles using the continuous-flow procedure. Symbols: triangle = azacytidine; square = RGU-CHO; rhombus = RGU; bold line = total azacytidine species.

The drug delivery profile obtained from the batch procedure is depicted in Fig. 6b. A faster release process during the first hours was obtained while the total concentration of drug species in the vessel increased moderately in the period from 2 to 7 h. We consider that the drug entrapped in the sample has been completely transferred to the solution in this process period. Although azacytidine and RGU-CHO were major components at the beginning of the process, concentrations of such species decayed markedly due to the occurrence of RGU degradation products. As mentioned, the main drawback of this batch strategy was the simultaneous occurrence of delivery and decomposition processes in the bulk of the solution. In order to solve this problem and facilitate the extraction of information, the continuous-flow methodology was introduced as a way of minimizing degradations.

The continuous-flow procedure was developed to simulate the behavior in the physiological environments in which fluids in contact with the product are continuously renewed. Here, a fresh 10 mM HCl solution was continuously pumped through the system at a constant flow rate of 1 ml min^{-1}, thus facilitating the transference of drug towards the liquid phase. The short residence time inside the vessel minimized the extension of decomposition processes so that the formation of RGU was found to be negligible. Cumulative release kinetics were constructed by integrating the drug amounts found in the solution fractions collected over time.

As shown in Fig. 6c, the delivery process at pH 2 occurred in 2 h, approximately, and a 100% of drug was released from the sample. It was also observed that the azacytidine/RGU-CHO concentration ratio was constant over time. The comparison of processes in Fig. 6a and c proved that the stability of the drug inside the coating was clearly superior to that of the pure drug. Note that azacytidine was the predominant species during the whole process, thus, suggesting that unaltered drug was continuously released to the medium. As a result, it was concluded that the polymeric coating reasonably protected azacytidine from a rapid hydrolysis and the active cytostatic compounds were dissolved in the medium until the drug contained in the sample run out.

3.4.2. Delivery at pH 7.4

The characterization of the release process at pH 7.4 was carried out in a similar way as described at pH 2. Hence, dissolution/stability of raw azacytidine and batch and continuous-flow release processes were studied (see results in Fig. 7). First, it was observed that the stability of azacytidine at this pH was slightly superior to that found at pH 2. Indeed the percentage of residual unaltered drug after 7 h was 15%.

Results of the drug release from the azacytidine–PLA sample obtained with the batch procedure are also given in Fig. 7b. In more detail, various chromatograms taken over time are depicted in Fig. 8 as an example for illustrating the delivery evolution over time. Peaks of azacytidine and RGU-CHO, referred to as 1 and 2, were the only present at the beginning of the process. These peaks

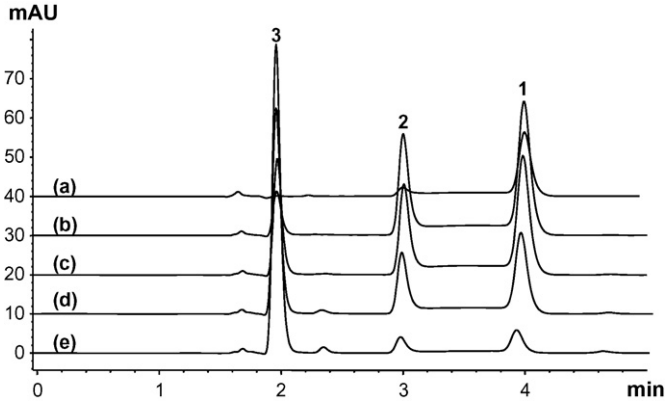

Fig. 8. Chromatogram study of the drug delivery process at pH 7.4 using the batch procedure. (a) Process time = 30 s; (b) process time = 1 h; (c) process time = 2 h; (d) process time = 3.5 h; (e) process time = 7. Peak assignment: 1 = azacytidine; 2 = RGU-CHO; 3 = RGU.

increased during the first hour of process due to the progressive release of the drug to the solution. At process times longer that 2 h, however, these peaks decreased due to their degradation to form RGU. It can be seen that RGU was the major component of the system after 7 h while the peaks of azacytidine and RGU-CHO were significantly smaller.

The release process was also checked by continuous-flow analysis and results obtained were similar to those found at pH 2. The release was faster than in batch experiments since the continuous renewal of the solution favored the lixiviation of the drug. Hence, the drug content was fully delivered in 2 h, approximately. Besides, results showed that RGU was undetectable in such conditions, thus indicating that it was formed later from the degradation of drug in the bulk solution. The stability of the drug entrapped in the polymer was again evidenced as unaltered azacytidine was the principal component released from the drug–PLA sample.

4. Conclusions

SAS has been found to be an efficient technique for preparing sustained delivery systems involving unstable polar drugs. In this case a high yield of product of more than 80% has been obtained from their ingredients, azacytidine and poly-lactic acid, with a final composition that maintain the original composition in the processed solution. Beside, the drug entrapment has been highly satisfactory, with a 95% of drug coated with a polymeric film. The release characteristics have been assessed in acid and slightly basic media (pH 2 and 7.4, respectively). In the continuous-flow procedure, the constant renewal of the release solution forces the drug to be delivered in 2 h, approximately. In this case, a 100% of delivery is achieved in both acid and basic media. Results have demonstrated that the stability of the drug in the azacytidine–PLA sample has been clearly improved with respect to the pure solution. Indeed, unaltered azacytidine is continuously released from the delivery system while the formation of degradation products occurs in the bulk of the solution.

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