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Short communication

Probing the release of the chronobiotic hormone melatonin from hybrid calcium alginate hydrogel beads

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A variety of commonly used hydrogels were utilized in the preparation of calcium alginate beads, which incorporate the chronobiotic hormone melatonin (MLT). The *in vitro* release of the hormone in aqueous media at pH 1.2 and 6.8 was probed in the conjunction with the swelling of the beads and their thermal degradation properties. It has been found that the release of MLT from the beads was reversibly proportional to the extent of their expansion, which depends on the molecular mass/viscosity of the biopolymers present in the beads; the higher the molecular mass/viscosity of the hydrogels the greater the beads swelling and the less the MLT's release. Thermogravimetric analysis (TGA) data support the presence of the components in the hybrid hydrogel beads and elucidate their effects on the thermal stability of the systems. Thus, the physicochemical properties of the biopolymers used, along with their stereoelectronic features modulate the release of MLT from the beads, providing formulations able to treat sleep onset related problems or dysfunctions arising from poor sleep maintenance.

Keywords: melatonin, hydrogels, calcium alginate beads, swelling behavior, thermogravimetric analysis, *in vitro* release

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Melatonin (*N*-acetyl-5-methoxytryptamine, MLT) is a hormone synthesized from the biogenic amine serotonin in the pineal gland (1). Norepinephrine also plays an important role in the production and the release of MLT, which is influenced by exposure to light (2). In addition, the necessary enzymes for the biosynthesis of MLT remain inactive in daylight and are activated during the night hours (3). The actions of MLT are associated with its binding to G-protein-coupled cell membrane receptors. So far, three different types of MLT's receptors have been characterized (MT1, MT2 and MT3) (4). MLT, a hormone involved in the regulation of the circadian rhythms, has therapeutic value in some blind subjects, restoring their disturbed circadian cycle (5). It also has a positive impact on the treatment of seasonal affective disorders (SAD), which afflict some people during the winter days (6), and it has been used to resynchronize the clock in jet-lag sufferers (7). MLT has

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also been implicated in a range of other diseases, including Parkinson's disease (8), Alzheimer's disease and other neurodegenerative conditions (9), and in various cancers (10). It has also been reported to be a potent antioxidant (11).

We have been interested in the development of dosage form products, which mimic the physiologically secreted high MLT levels at night, necessary to promote sleep onset and maintain sleep (12). Sleep dysfunctions, referring to sleep onset difficulties, are better treated with fast release MLT products, whilst those related to sleep maintenance problems are preferably treated with MLT modified release formulations (13–21). In this report we communicate our findings on the *in vitro* release of MLT from calcium alginate (CA) hydrogel beads, containing combinations of polyvinylpyrrolidone (PVP_{10000} and PVP_{55000}), hydroxypropylmethylcellulose ($HPMC_{15000}$ and $HPMC_{100000}$), lactose monohydrate and as a surfactant sodium lauryl sulfate (SLS).

Beads are used for the modified release of vaccines, antibiotics, hormones and other drugs of diverse structures, as they provide large surface areas and possess an easier estimation of diffusion and mass transfer behavior. Furthermore, polymeric beads show high bioavailability and stability. Other advantages include limited fluctuation in the therapeutic range, reduced side effects, decreased dosing frequency and improved patient compliance (21, 22). Moreover, the swelling behavior in conditions, like those of gastrointestinal liquids, is a very important parameter that has to be investigated during the development of these systems for the oral administration of drugs (23). These compelling characteristics of the beads intrigued us to utilize them in our attempt to develop MLT formulations that can either treat sleep onset or poor sleep maintenance related problems. It was found that the higher the molecular mass/viscosity of the biopolymer, used in the construction of the beads, the higher their expansion leading to lower and prolonged MLT release. Thus, the physicochemical properties of the hydrogel used can modulate the release of MLT from the CA beads, resulting to formulations that have the potential to treat sleep onset related problems formulations (CA:PVP_{10000}, CA:PVP_{10000}:$HPMC_{15000}$ 1, CA:PVP_{55000}, CA:Lactose and CA:PVP_{10000}:Lactose) and to formulations suitable for alleviating sleep maintenance dysfunctions formulations (CA:$HPMC_{15000}$, CA:PVP_{10000}:$HPMC_{15000}$ 2, CA:PVP_{55000}:$HPMC_{15000}$, CA:$HPMC_{100000}$).

To the best of our knowledge, this is the first report in the literature, where CA beads and mixtures of polymers are used for the encapsulation of MLT. The prepared systems were found to be suitable for the pH-responsive release of MLT in a manner strongly dependent on the formulation of the systems. This study could serve as a roadmap for the design and development of formulations with controlled-release properties.

EXPERIMENTAL

Materials

All of the materials were of analytical grade and used as received without further purification. In detail, MLT (M_r = 232.28; melting point 117 °C, solubility in water 2 g L^{-1} at 20 °C and 5 g L^{-1} at 50 °C and octanol/water partition coefficient 1.6) was purchased from the MP Biomedicals LLC (France). Low viscosity (250 mPa s of 2 % solution) alginic acid sodium salt (NaAlg) and calcium chloride dihydrate ($CaCl_2 \times 2\ H_2O$) were purchased from Sigma-Aldrich (USA). Furthermore, the simulated gastric fluid (SGF, pH 1.2) and the simu-

lated intestinal fluid (SIF, pH 6.8) were prepared as described in the USP29-NF24 Pharmacopeia (24). Macrogol 400 (PEG 4000) was given by Fagron Hellas (Greece) and lactose monohydrate was obtained from Merck. Polyvinylpyrrolidone (PVP_{10000}, low Mw and PVP_{55000}, high Mw), hydroxypropylmethylcellulose ($HPMC_{15000}$, low Mw and $HPMC_{100000}$, high Mw) and sodium lauryl sulfate were purchased from Sigma-Aldrich. All of these materials were of analytical grade and used as received without further purification.

Preparation of CA MLT beads

MLT (2 mg) was dissolved in a solution containing 0.4 mL PEG 4000, 0.6 mL H_2O and 0.025 mg of SLS, and then added to the alginate solution (*vide infra*).

The protocol reported by Pasparakis *et al.* (25), was followed for the preparation of the CA beads. Detailed experimental for the formation of the particular beads is given in the Supplementary Information file. Table I summarizes the bead compositions.

Table I. Composition of CA MLT beads

Formulation	MLT (mg)	PVP_{10000} (mg)	Lactose monohydrate (mg)	PVP_{55000} (mg)	$HPMC_{15000}$ (mg)	$HPMC_{100000}$ (mg)
CA/MLT	2					
CA/PVP_{10000}	2	35				
$CA/HPMC_{15000}$	2				35	
$CA/PVP_{10000}/HPMC_{15000}$ 1	2	15			20	
$CA/PVP_{10000}/HPMC_{15000}$ 2	2	5			30	
$CA/PVP_{55000}/HPMC_{15000}$	2			5	30	
$CA:PVP_{55000}$	2			35		
$CA:HPMC_{100000}$	2					35
CA/Lactose	2		35			
CA/PVP_{10000}/Lactose	2	17.5	17.5			

Thermogravimetric analysis

A TA Instrument Q500 TGA Analyzer was utilized for the thermogravimetric analysis (TGA) experiments, in the temperature range of 30–900 °C, at a heating rate of 10 °C min^{-1} in an air atmosphere. Dry beads were weighed directly into the TGA pan.

Entrapment efficiency

The MLT Entrapment Efficiency (EE%) of the beads was calculated by measuring the absorbance of the supernatant solution obtained from the entrapment medium after centrifugation (Sigma 3-0KS, Germany), employing a Perkin-Elmer UV spectrophotometer

(Norwalk, CT) at 278 nm and determining the EE% from Equation *1*. MLT concentration was determined from the MLT standard plot.

$$\text{EE\%} = \frac{\text{Total melatonin added} - \text{Nonentrapped melatonin}}{\text{Total melatonin added}} \times 100 \qquad (1)$$

Swelling studies and measurements

The beads were spread on aluminum foil and dried at 40 °C overnight in an oven (WTB B34 Binder GmbH, Germany). Dry beads were used for the swelling studies. The swelling experiments were performed in two discrete dispersion media, in accordance with the reported protocols (26, 27). Full experimental details are given in the Supplementary Information file.

The dynamic mass change (%) of the beads with respect to time was calculated according to Equation *2*:

$$\text{Mass change} = \frac{m_t - m_i}{m_i} \times 100 \qquad (2)$$

where m_t is the mass of the swollen beads and m_i is their initial mass.

MLT release studies

A USP XXII dissolution apparatus II (Pharma test, Germany) (paddle method) was employed for determining MLT's release from the prepared formulations. The beads were placed into two different media simulating, in subsequent order, the stomach and the enteric environment. At the end of the first phase (450 mL HCl (1 mol L^{-1}), pH 1.2 for the first 120 min), a solution of 450 mL K_2HPO_4 (0.14 mol L^{-1}), pH 9, was added to acquire the desired composition of the next phase (final volume 900 mL, pH 6.8) and the resulting mixture was maintained at a controlled temperature of 37.0 ± 0.5 °C. The paddles' rotation was set at 50 rpm. At each time point, the samples were removed and replaced with an equal volume of fresh medium. The concentration of MLT released into the medium was recorded using a Perkin-Elmer UV spectrophotometer (USA), at a wavelength of 278 nm.

Release kinetics

In order to decipher the drug release mechanisms, the dissolution test results were analyzed using the Korsmeyer-Peppas equation (*3*):

$$\frac{M_t}{M_\infty} = kt^n \qquad (3)$$

where M_t/M_∞ is the fraction of drug released at time t, k is the constant related to the structural and geometric characteristic of the device, and n is the release exponent, indicative of the drug release mechanism. The k and n values were measured up to the initial 40 % release of the drug. In the case of spheres, values of n between 0.43 and 0.85 are indicative of both diffusion-controlled drug release and swelling controlled drug release. Values higher than 0.85 indicate Case-II transport, which during gel swelling, is related to polymer relaxation (28, 29).

Statistical analysis

In order to compare the dissolution profiles, graphs of MLT (%) release (mean ± SD) *vs.* time were produced and the Dissolution Efficiency (DE%) (30) was calculated.

Initially, descriptive statistics were applied to the swelling and DE% data. The normality of distribution was assessed using the nonparametric Shapiro-Wilk test and QQ plots. Based on these results, the parametric one-way analysis of variance (ANOVA) was applied separately to the swelling and DE% data using the term 'Formulation' as a grouping factor. Post hoc tests, using the Tukey statistic, were also performed to identify potential differences between the formulations. In all analyses, the significance limit was set at 5 % (*i.e.*, $p = 0.05$). The entire analysis was implemented in IBM SPSS v.24 (IBM Corp, USA).

RESULTS AND DISCUSSION

Thermogravimetric analysis

The degradation process of CA and their hybrid analogues is depicted in Fig. 1. A certain pattern of seven degradation processes and one plateau is observed in all thermogravimetry graphs, with differences in the temperature range for each sample. The first degradation is observed in the range of 30–200 °C, which is due to the loss of physisorbed and chemisorbed water molecules. The second three-step degradation process occurs in the 190–220 °C, 220–260 °C and 260–290 °C temperature ranges resulting in a 40–45 % mass loss, due to the elimination of hydroxyl groups and alginate backbone degradation (31). These procedures were slightly transposed to higher temperatures for the CA/HPMC samples. They also present two more degradation processes at the temperature range of 350–380 °C and 380–500 °C, that are due to the melting and degradation of HPMC, respectively (32). The CA/PVP samples follow the same degradation path as the plain CA samples in the temperature range of 30–400 °C. A slight differentiation for the CA/PVP_{55000} sample is noticed in the range of 300–400 °C since it exhibits a 4 % mass loss in comparison with the CA/PVP_{15000} sample. The

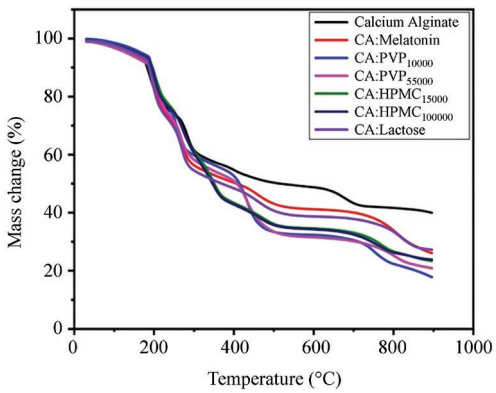

Fig. 1. Thermogravimetric analysis curves for all calcium-alginate beads (CA – calcium alginate, HPMC – hydroxypropylmethylcellulose, PVP – polyvinylpyrrolidone). The axes denote the mass change (%) *vs.* temperature (°C).

further temperature increase in the range of 400–500 °C leads to a 20 % mass loss for both CA/PVP samples, due to the full degradation of the PVP polymer (33). The CA/MLT and the CA/Lactose samples present a two-step process in the range of 300–500 °C, with an observed mass loss of 18 % for CA/MLT and 20 % for CA/Lactose respectively, depicting the decomposition of MLT and lactose (34). The final degradation process for the CA sample occurs in the region of 650–750 °C and is a result of decarboxylation and the formation of calcium oxide and calcium hydroxide (31). On the other hand, a certain hysteresis of the latter phenomenon is observed for the hybrid samples, bringing the particular process at higher temperatures of at least 50 °C. More specifically, the CA/PVP_{10000} and the two CA/HPMC samples present the final two-step decomposition at 700 °C, while the final one-step CA/MLT, CA/Lactose and CA/PVP_{55000} degradation takes place at 750 °C.

Entrapment efficiency of MLT beads (EE%)

The EE% values obtained for all beads' formulations, containing 2 mg of MLT, were similar, as shown in Table II. In all cases the rate of EE exceeded 78 %, showing that the method followed for the entrapment of MLT in the beads is effective.

Table II. Entrapment of MLT in different formulations (EE%)

Formulation	Entrapment efficiency (EE%)
CA/MLT	78.83 ± 1.04
CA/PVP_{10000}	82.17 ± 1.61
CA/$HPMC_{15000}$	78.67 ± 1.44
CA/PVP_{10000}/$HPMC_{15000}$ 1	78.83 ± 2.08
CA/PVP_{10000}/$HPMC_{15000}$ 2	79.00 ± 1.32
CA/PVP_{55000}/$HPMC_{15000}$	79.17 ± 1.53
CA/PVP_{55000}	79.50 ± 10.50
CA/$HPMC_{100000}$	78.17 ± 0.76
CA/Lactose	79.17 ± 1.04
CA/PVP_{10000}/Lactose	81.83 ± 1.04

The indicated values are the mean of 3 measurements ± SD.

Swelling studies

The swelling profile of the dry beads in SGF (< 2 h), and in SIF, thereafter, is demonstrated in Fig. 2. The swelling of the biopolymers is presented in the graphs of mass change (%) *vs*. time; the swelling was found to be dependent on the aqueous medium. The CA containing beads swell considerably in SGF (pH = 1.2) and to a lesser extent in the SIF (pH = 6.8) medium. The increase in the beads' mass reaches ~350 %. This increase is due to the hydration of the carboxylic acid functional groups (35).

The descriptive statistical criteria of swelling at 180 min are listed in Table S1 in the supplementary information. Further analysis of swelling (at 180 min) resulted in statistical

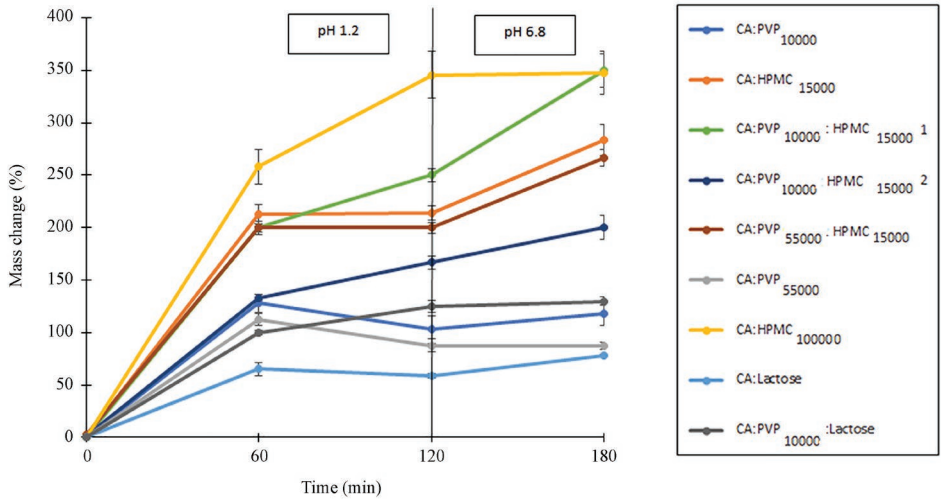

Fig. 2. Swelling profiles of dry beads at pH 1.2 for the first two hours and at pH 6.8, thereafter. The results denote the mean ± S.D. ($n = 3$) (CA – calcium alginate, HPMC – hydroxypropylmethylcellulose, PVP – polyvinylpyrrolidone).

significant differences ($p < 0.05$) among all pairs of formulations (Table S2) except from the following: CA/PVP$_{10000}$ *vs.* CA/PVP$_{55000}$, CA/PVP$_{10000}$ *vs.* CA/PVP$_{10000}$/Lactose, CA/HPMC$_{15000}$ *vs.* CA/PVP$_{55000}$/HPMC$_{15000}$, CA/PVP$_{10000}$/HPMC$_{15000}$ *vs.* CA/HPMC$_{100000}$, and CA/PVP$_{55000}$/HPMC$_{15000}$ *vs.* CA/HPMC$_{15000}$.

MLT release-studies

It is evident, from the MLT loaded hydrogel CA beads swelling data that the extent of their expansion is pH-dependent and related to the nature and the molecular mass/viscosity of the biopolymer used (Fig. 2). Thus, the MLT beads containing HPMC$_{100000}$ showed the highest swelling (formulation CA/HPMC$_{100000}$), which was followed by their congeners containing HPMC 15000 (formulation CA/HPMC$_{15000}$). The same trend was observed, but to a lesser extent, in the case of the PVP containing MLT beads (formulation CA/PVP$_{55000}$ *vs.* formulation CA/PVP$_{10000}$). The lactose monohydrate MLT beads (formulation CA/Lactose) swelled less than all the above systems. It is well documented that the higher the molecular mass/viscosity of HPMC and PVP the higher their swelling due to the thicker gel they form, when in contact with aqueous media (36, 37).

The release of MLT from the beads was found to be reversibly proportional to the molecular mass/viscosity of the HPMC hydrogels they contain (Fig. 3). Thus, in the acidic pH medium, the release of MLT from the HPMC$_{15000}$ beads is substantially higher (formulation CA/HPMC$_{15000}$ 97.03 % at $t = 120$ min) than the respective release from the HPMC$_{100000}$ containing beads (formulation CA/HPMC$_{100000}$ 86.87 % at $t = 120$ min). A similar MLT release profile emerged and in the case of the different molecular mass/viscosity PVP containing beads (formulation CA/PVP$_{10000}$ 93.24 % at $t = 60$ min *vs.* formulation CA/PVP$_{55000}$ 73.37 % at $t = 60$ min). At pH 6.8, the same trend was observed in both the HPMC and PVP

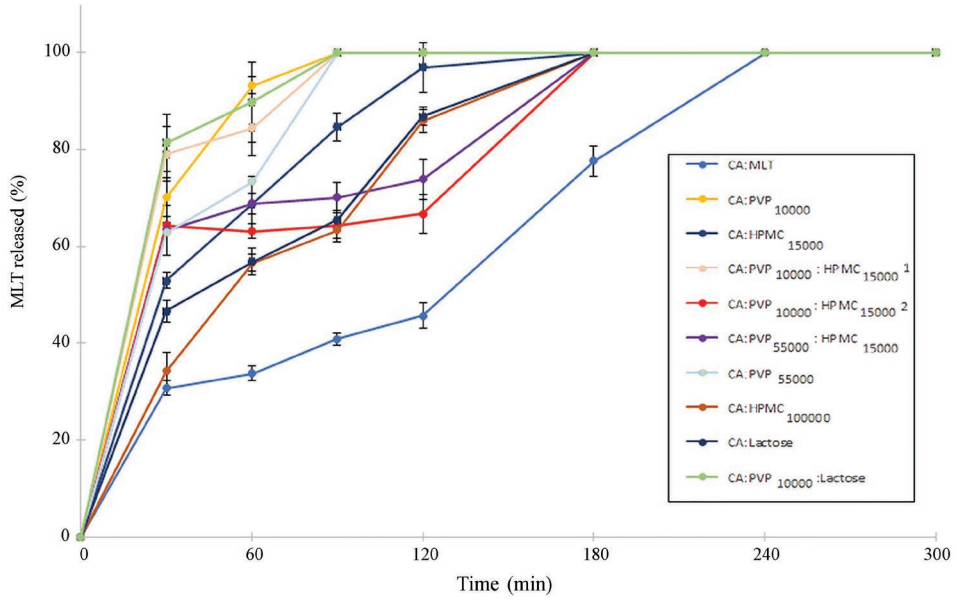

Fig. 3. *In vitro* MLT release from different CA beads formulations. The results denote the mean ± S.D. ($n = 3$) (CA – calcium alginate, HPMC – hydroxypropylmethylcellulose, PVP – polyvinylpyrrolidone).

cases, but it is of no practical meaning as almost the entire amount of MLT was already released in the acidic pH medium, at $t \leq 120$ min.

Moreover, from the mechanistic point of view at a molecular level, and besides the influence of the extent of swelling caused by the two different types of HPMC, present in the beads, on MLT's release, the fact that a large number of HPMCs CH_2OH groups are methylated, reduces the ability of these hydrogels to form hydrogen bonds with MLT's C5-OMe. This results in the reduced solubility of the hormone noticed in this case.

Conversely, the facile solubilization of MLT from the PVP containing CA beads can be attributed to the conversion of PVP (**II**) to the *gem*-diol (**III**), at pH 1.2 (38). The hydroxyl groups of **III** participate in H-bond formation with MLT's C5-oxygen atom and the $NHCOCH_3$ group, thus facilitating its aqueous solubility.

With respect to the release kinetics, it was found that the release of MLT in the case of formulations 1 and 9 followed a Fickian diffusion mechanism ($n = 0.31$). The value of the diffusion coefficient ($n = 0.53$), in the case of the $CA/HPMC_{100000}$ formulation, is an indication of both diffusion-controlled drug release and swelling-controlled drug release kinetics (anomalous transport). The diffusion coefficient n values could not be calculated in all other cases, as the release exceeded 60 %, at cca $t = 30$ min.

The extent of the expansion of the CA beads containing mixtures of hydrogels (formulations $CA/PVP_{10000}/HPMC_{15000}$ 1, $CA/PVP_{10000}/HPMC_{15000}$ 2, $CA/PVP_{55000}/HPMC_{15000}$ and CA/PVP_{10000}/Lactose) was found to depend on the nature of the respective biopolymers and their relevant concentration in the mixtures. Thus, the least bead expansion was

seen in the case of formulation CA/PVP_{10000}/Lactose, where the moderately swelling promoter, lactose monohydrate, was co-present with PVP_{10000}, in the mixture, in the same amount (17.5 mg). A high bead's expansion was noticed in the case of formulation CA/PVP_{10000}/$HPMC_{15000}$ 2, where $HPMC_{15000}$ (30 mg) was mixed with a smaller amount of PVP_{10000} (5 mg). Even higher was the expansion of the beads of formulation CA/PVP_{55000}/$HPMC_{15000}$, as these were prepared by mixing 5 mg of PVP_{55000} and 30 mg of $HPMC_{15000}$. The formulation CA/PVP_{10000}/$HPMC_{15000}$ 1, CA beads, containing $HPMC_{15000}$ (20 mg) and PVP_{10000} (15 mg) swelled more than all the rest mixture beads composites.

Once again, the release of MLT was affected by the relevant concentration and nature of the hydrogels in the mixture. Thus, in the acidic pH medium the release of MLT from formulation CA/PVP_{10000}/$HPMC_{15000}$ 2 (30 mg of $HPMC_{15000}$ and 5 mg of PVP_{10000}) is much lower (66.73 %) than from formulation CA/PVP_{10000}/$HPMC_{15000}$ 1 (20 mg $HPMC_{15000}$ and 15 mg PVP_{10000}). An analogous MLT release pattern was observed in the case of formulation CA/PVP_{55000}/$HPMC_{15000}$ (30 mg of $HPMC_{15000}$ and 15 mg of PVP_{55000}). These differences in the release of MLT from mixtures of biopolymers is more profound in the case of formulation CA/PVP_{10000}/Lactose, where MLT is entrapped in lactose monohydrate and PVP_{10000} constructed beads. In this case, the release of MLT reached 100 % at t = 90 min. In the pH 6.8 medium, at $t \geq 120$ min, an analogous MLT release pattern was observed.

As it was previously mentioned, we aimed at designing and developing dosage forms, which mimic the physiologically secreted high MLT levels at night, necessary to promote sleep onset and maintain sleep. From the results obtained from the release studies, it became apparent that in all cases, MLT released from the prepared beads (40–80 %) during the first 30 min. This release profile is useful for the promotion of sleep. In other words, insomniacs may use the particular dosage form 30 min before bedtime. Moreover, the remaining MLT quantity is released within the next 3 h, and thus these formulations are also useful in maintaining sleep.

Table S3 lists the descriptive statistical criteria of DE%. Additional statistical analysis of the DE% values among the various formulations is presented in Table S4 (Supplementary Information).

CONCLUSIONS

Several hybrid CA hydrogel beads containing polymeric biomaterials and MLT as the active pharmaceutical ingredient have been produced and studied. It was shown that the right choice of common polymeric hydrogels, in terms of the nature of the polymer, its molecular mass and the composition for the preparation of CA MLT beads, determines the encapsulation and swelling properties of the hybrid beads. The successful formulations may be effectively used for treating circadian rhythm desynchronization disorders. As a result, these MLT formulations can either treat sleep onset or poor sleep maintenance related problems.

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