JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

A novel formulation of zolpidem for direct nose-to-brain delivery: synthesis, encapsulation and intranasal administration to mice

Tatiana Borodina[a,b,*], Irina Marchenko[a,c,*], Daria Trushina[a,b,c,*], Yulia Volkova[d], Valerii Shirinian[d], Igor Zavarzin[d], Evgeny Kondrakhin[e], Georgy Kovalev[e], Mikhail Kovalchuk[a,c] and Tatiana Bukreeva[a,c]

[a]Shubnikov Institute of Crystallography of Federal Scientific Research Centre "Crystallography and Photonics" of Russian Academy of Sciences, Moscow, Russia, [b]Institute of Molecular Medicine, Sechenov First Moscow State Medical University, Moscow, Russia, [c]National Research Centre "Kurchatov Institute", Moscow, Russia, [d]N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia and [e]Zakusov Institute of Pharmacology, Moscow, Russia

Keywords
anxiolytic effect; intranasal delivery; microcontainers; zolpidem

Correspondence
Tatiana Borodina, Shubnikov Institute of Crystallography of Federal Scientific Research Centre "Crystallography and Photonics" of Russian Academy of Sciences, Leninsky Prospect, 59, Moscow 119333, Russia.
E-mail: tatiana_borodina@hotmail.com

Received December 25, 2017
Accepted June 3, 2018

doi: 10.1111/jphp.12958

*Tatiana Borodina, Irina Marchenko and Daria Trushina contributed equally to this work.

Abstract

Objectives Anxiolytic drug zolpidem was incorporated into the microcontainers based on mesoporous calcium carbonate particles modified by diethylaminoethyl-dextran/hyaluronic acid shell. The release of zolpidem in saline solution and in polymer film modelling nasal mucosa was investigated. The anxiolytic effect of zolpidem upon intranasal administration of microcontainers and free medicine was determined by *in vivo* experiments on mice.

Methods The structures of all compounds during zolpidem synthesis were established using nuclear magnetic resonance spectroscopy. The loading efficacy and release kinetics of zolpidem were analysed by spectrophotometry. Surface morphology of formulation was investigated by scanning electron microscopy. To determine the effect of zolpidem-loaded containers administration by the intranasal route *in vivo* experiments was carried out applying the open field test.

Key findings Nasal administration of zolpidem in the form of the microcontainers based on mesoporous calcium carbonate particles modified by diethylaminoethyl-dextran/hyaluronic acid shell has a pronounced anxiolytic effect on the behaviour of the animals in the open field test.

Conclusions The polyelectrolyte shell deposited together with zolpidem enhances the loading efficacy of the microcontainers. *In vivo* experiments on mice demonstrate increase in anxiolytic effect of zolpidem in microcontainers compared with upon intranasal administration of free medicine.

Introduction

Lifestyle of the modern people often causes everyday stress provoking daytime problems including sleepiness that can contribute to anxiety, irritability, concentration problems, memory problems, poor immune system function and reduced reaction time.[1,2] Untreated insomnia can progress into chronic form and impact to a general feeling of being unwell both mentally and physically.[3,4] All these result in multiple daytime consequences that can lead to depression, alertness and lowering of cognitive abilities.

Nowadays, various pharmacological agents are used as an anxiolytic drugs and for the insomnia treatment. First-generation hypnotics (barbiturates, carbamates, chloral hydrate and methaqualone) induce a prolonged hypnosedative effect, resulting in working daytime impairment of the patients.[5] Benzodiazepine drugs were proposed as the second-generation hypnotics and possess minor side effects and toxicity. However, protracted application of benzodiazepines provides ineligible side effects including rebound insomnia.

In the last decades, a new generation of non-benzodiazepine drugs – Z-drugs, which are widely used for the treatment of insomnia, was emerged. Among them, zolpidem (imidazopyridine) has become the most popular medicine for this disease. Imidazopyridines could be useful in the

management of short-term insomnia, but not for maintaining sleep through the night. Besides hypnotic effect zolpidem also possesses anxiolytic properties. For example, anxiolytic-like activity of zolpidem in mice was demonstrated by Griebel.[6] The clinical study showed that zolpidem facilitates improving anxiety states of insomniac patients.[7] In order to broaden the zolpidem action in the treatment of different types of insomnia and depression, the medicine could be incorporated into polymeric delivery systems. Al-Dhubiab demonstrated an entrapment of zolpidem into poly(lactic-co-glycolic acid) nanospheres that can provide a rapid onset of action together with prolonged release of the drug.[5] Trapani *et al.*[8] have also incorporated the hypnotic agent zolpidem in the microparticles constituted by poly(DL-lactide) and poly(DL-lactide-co-glycolide). Both approaches are based on the emulsion methods using organic solvents.

In the present paper, we have proposed mesoporous microcontainers based on biocompatible calcium carbonate particles. The microparticles were synthesized by simple and reproducible procedure without applying hazardous solvents and chemicals. The porous structure of the calcium carbonate ensures entrapment of the drug in the final formulation. The freshly synthesized zolpidem was encapsulated into the microcontainers formed by the electrostatic adsorption of polyelectrolytes onto surface of the microparticles.[9–11] We studied encapsulation efficacy and release of zolpidem. The microcontainers are proposed for intranasal delivery of the encapsulated compounds.

In the previous study, we demonstrate the high efficiency of the microcontainers based on calcium carbonate particles to the brain delivery of central anesthetic loperamide upon their intranasal administration.[12,13] Current research demonstrates an effective nasal delivery of sedative agent zolpidem that could be useful in the treatment of various anxiety disorders. The microcontainers administrated nasally could extend an activity of zolpidem and prolong its anxiolytic action.

Previously, it was demonstrated that $CaCO_3$ powder formulation increases the nasal bioavailability of hydrophilic compounds in a range of molecular weights presumably through retardation of the drug outflow from the nasal cavity.[14] The intranasal administration has the great potential advantage for the rapid drug delivery to the central nervous system together with non-invasiveness and fast onset of medicine therapeutic effect. So, in work[15] pharmacokinetic studies in rats demonstrated that intranasally administered zolpidem could achieve significantly faster absorption rate and higher plasma concentration than that by oral route. Application of polymer vehicles for the intranasal administration can impart several advantages to the drug formulation, such as increase in the residence time in nasal cavity (delay of mucacellular clearance followed by prolonged drug adsorption and more sufficient pharmacologic effect), high bioavailability and easiness of use.

Experimental Section

Materials

Diethylaminoethyl-dextran (DEAE-D, MW 150 kDa) was obtained from SERVA, Germany. Hyaluronic acid (HA, MW 200–300 kDa), alginic acid sodium salt (15–25 cP, 1% in H_2O), sodium carbonate, sodium chloride, calcium chloride, sodium bicarbonate, toluene, ethyl alcohol, 5-methyl-2-aminopyridine, 4- methylbenzaldehyde and dichloromethane were purchased from Sigma-Aldrich and used without any further purification unless otherwise noted. Zolpidem was used as water-soluble tartrate. Toluene, ethyl alcohol and dichloromethane were dried and purified under nitrogen using standard methods and were distilled immediately before use.[16] Parent (E)-5-methyl-N-(4-methylbenzylidene)pyridin-2-amine was prepared according to published procedure from the 4-methylbenzaldehyde and 5-methyl-2-aminopyridine.[17]

Methods

The structures of all compounds were established using 1D NMR (1H, 13C) spectroscopy. NMR spectra were acquired on Bruker Avance 300 spectrometer at room temperature; the chemical shifts δ were measured in ppm relative to the solvent (1H: CDCl3, $\delta = 7.27$ ppm; 13C: CDCl3, $\delta = 77.00$ ppm). Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, double doublet; ddd, double double doublet. The coupling constants (J) are in Hertz. High-resolution and accurate mass spectra were obtained on BrukermicrOTOF-QTM ESI-TOF (electrospray ionization (ESI)/Time of Flight) and Thermo Scientific* LTQ Orbitrap mass spectrometers. IR spectra were recorded on a Specord M-80 instrument in KBr pellets. Melting points (mp) are uncorrected and were measured on a Boetius capillary mp apparatus. Analytical thin layer chromatography (TLC) was carried out on silica gel plates (silica gel 60 F254 aluminium supported plates); the visualization was accomplished with an UV lamp (365 nm). Column chromatography was performed on silica gel 60 (230–400 mesh; Merck & Co., Kenilworth, NJ, USA) treated with 1% triethylamine solution in petroleum benzene.

The loading efficacy and release of zolpidem were analysed by spectrophotometer Perkin Elmer Lambda 650. Surface morphology was investigated by scanning electron microscope Jeol JSM-7401F.

High-resolution mass spectra (HRMS) were measured using ESI.[18] The measurements were carried out in

positive ion mode (interface capillary voltage 4500 V); the mass ratio was from m/z 50 to 3000 Da; external/internal calibration was performed using an electrospray calibrant solution. Syringe injection was used for solutions in CH_3CN (low rate 3 ml/min). Nitrogen was applied as a dry gas and the interface temperature was set at 180°C. Mass spectra were recorded on a Finnigan Mat INCOS 50 Quadrupole Mass Spectrometer at 70 eV. Melting points are uncorrected and were measured on a Boetius capillary melting point apparatus. Analytical TLC was carried out on silica gel plates (silica gel 60 F254 aluminium supported plates); the visualization was accomplished with an UV lamp (365 nm). Column chromatography was performed on silica gel 60 (230–400 mesh; Merck & Co., Kenilworth, NJ, USA) treated with 1% triethylamine solution in petroleum benzene. Alkynes, aldehydes and 2-aminopyridines were commercially available, as well as copper(I,II) salts, and were used without additional purification. All reactions were carried out using freshly distilled and dry solvents. Parent N,N-dipropylpropiolamide was prepared according to published procedures.[19]

Synthesis of zolpidem

Synthesis of zolpidem was achieved by modified protocols.[20–22]

5-Methyl-N-(4-methylbenzylidene)pyridin-2-amine (1)

The mixture of toluene aldehyde (6.6 ml, 0.055 mol) and 5-methyl-2-aminopyridine (5.4 g, 0.050 mol) was kept at room temperature overnight. Precipitate formed was particulated and vigorously stirred with 10 ml of Et_2O for 1 h. The precipitate was separated by filtration, residue washed with Et_2O (2 × 5 ml) and dried at 60°C under vacuum to give 7.2 g (67% yield) product **1** as pale yellow solid. mp 119–120°C; R_f = 0.51 (petroleum ether - EtOAc, 5 : 1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.12 (s, 1H, CH=N), 8.31 (s, 1H, Py), 7.88 (d, *J* = 8.07 Hz, 2H), 7.54 (d, *J* = 9.53 Hz, 1H, Py), 7.25 (d, *J* = 8.07 Hz, 2H), 7.22 (d, *J* = 9.53 Hz, 1H, Py), 2.42 (s, 3H, Me), 2.36 (s, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.1 (CH), 159.2 (C), 149.1 (CH), 145.9 (CH), 142.4 (C), 133.6 (C), 131.4 (C), 129.6 (2 × CH), 129.5 (2 × CH), 119.3 (CH), 21.8 (CH_3), 15.9 (CH_3); HRMS (ESI) 211.1230, calcd. for $C_{14}H_{15}N_2^+$ 211.1230.

Ethyl 2-(6-methyl-2-(p-tolyl)imidazo[1,2-a]pyridin-3-yl)acetate (3)

The mixture of imine **1** (2.1 g, 0.01 mol), CuCl (26.0 g, 0.26 mol) and ethyl propiolate **2** (0.98 ml, 0.01 mol) in dry toluene (20 ml) was refluxed under inert over 1 h. Resulting mixture was deluted with EtOAc (20 ml) and washed with ammonium hydroxide solution (28% NH_3 in H_2O, 3 × 20 ml). Organic fraction was dried over Na_2SO_4 and solvent was removed under reduced pressure. Product was purified by column chromatography on silica gel to give 2.3 g (75% yield) of imidazopyridine **3** (eluent: Et_3N: EtOAc:petroleum ether 1 : 40 : 40).

Yellow solid, mp 85–99°C; R_f = 0.20 (petroleum ether – EtOAc, 3 : 1). The spectral data and melting point matched that reported by Namboothiri and coworkers.[21] ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (s, 1H), 7.74 (d, *J* = 8.07 Hz, 2H), 7.62 (d, *J* = 8.80 Hz, 1H), 7.29 (d, *J* = 8.07 Hz, 2H), 7.11 (d, *J* = 8.80, 1H), 4.24 (q, *J* = 7.39 Hz, 2H), 4.02 (s, 2H), 2.41 (s, 3H), 2.38 (s, 3H), 1.29 (t, *J* = 7.39 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.6 (CO), 144.5 (C), 144.0 (C), 137.6 (C), 131.4 (C), 129.4 (2 × CH), 128.4 (2 × CH), 127.6 (CH), 122.0 (C), 121.4 (CH), 116.8 (CH), 112.4 (C), 61.5 (CH_2), 30.9 (CH_2), 21.3 (CH_3), 18.4 (CH_3), 14.2 (CH_3); IR (KBr) 3429 (w), 1722 (s), 1185 (w), 789 (w) cm^{-1}; MS (ESI) 308 $[M]^+$.

Potassium 2-(6-methyl-2-(p-tolyl)imidazo[1,2-a] pyridin-3-yl)acetate (4)

KOH solution (0.8 g, 14.29 mmol) in EtOH (10 ml) was added to solution of imidazopyridine 3 (1.3 g, 3.68 mmol) in EtOH (20 ml) at room temperature under stirring. The stirring was continued till the starting material completely disappeared (10–15 min). Then activated carbon (200 mg) was added and the reaction mixture was stirred for 10–15 min. The resulting mixture was filtered through a pad of celite and the solvent was evaporated in vacuo. The residue was dissolved in water (10 ml) and extracted with CH_2Cl_2 (3 × 10 ml). The crude solution of potassium salt **4** was directly used for the next step without purification.

2-(6-Methyl-2-phenylimidazo[1,2-a]pyridin-3-yl) acetic acid (5)

Acetic acid (0.8 ml) was added to a stirred solution of salt 4 in water (10 ml) at room temperature. Then acetone (1–2 ml) was added and reaction mixture was kept over 0–10°C for additional 30 min. Precipitate formed was washed with water (3 × 5 ml), acetone (5 ml) and dried at r.t. to give 0.69 g (67% yield) of pure acid **5**.

White solid, mp 220–223°C; R_f = 0.42 (CH_2Cl_2 – MeOH, 4 : 1). The spectral data and melting point matched that reported by Namboothiri and coworkers.[21] ^{1}H NMR (300 MHz, DMSO-d6): δ 8.14 (s, 1H, Py), 7.69 (d, *J* = 8.06 Hz, 2H), 7.46 (d, *J* = 8.80 Hz, 1H, Py), 7.24 (d, *J* = 8.07 Hz, 2H), 7.10 (d, *J* = 8.80, 1H, Py), 2.33 (s, 3H), 2.28 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d6): δ 171.6

(CO), 142.8 (C), 141.9 (C), 136.5 (C), 132.0 (C), 129.1 (2 × CH), 127.8 (2 × CH), 127.1 (CH), 122.4 (C), 120.8 (CH), 116.5 (C), 115.9 (CH), 31.9 (CH_2), 20.9 (CH_3), 17.9 (CH_3); MS (ESI) 280 $[M]^+$.

N,N-dimethyl-2-(6-methyl-2-(p-tolyl)imidazo[1,2-a]pyridin-3-yl)acetamide (7, Zolpidem)

PCl5 (1.0 g, 4.92 mmol) was added to a stirred suspension of imidazopyridine carboxylic acid 5 (0.69 g, 2.46 mmol) in CH_2Cl_2 (20 ml) and the reaction mixture was kept at room temperature till the starting compound was completely consumed (monitoring by TLC). Then the reaction mixture was cooled to 0°C and dimethylamine gas (excess) was bubbled through it. The stirring was continued till the completion of the reaction (30–45 min). The reaction mixture was washed with ice-water (3 × 6 ml) and dried over Na_2SO_4. Solvent was removed under reduced pressure and the residue was washed with Et_2O (3 × 5 ml) to get pure amide **7** (0.42 g, 56% yield).

White solid, mp 195–198°C; R_f = 0.20 (petroleum ether – EtOAc, 1 : 3). The spectral data and melting point matched that reported by Namboothiri and coworkers.[21] ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (s, 1H, Py), 7.61 (d, J = 8.06 Hz, 2H), 7.48 (d, J = 8.80 Hz, 1H, Py), 7.24 (d, J = 8.07 Hz, 2H), 7.07 (d, J = 8.80, 1H, Py), 2.94 (s, 3H), 2.87 (s, 3H), 2.37 (s, 3H), 2.31 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.6 (CO), 144.1 (C), 144.0 (C), 137.5 (C), 132.0 (C), 129.1 (2 × CH), 127.8 (2 × CH), 127.5 (CH), 122.4 (C), 121.8 (CH), 116.5 (C), 113.8 (CH), 37.7 (CH_3), 36.0 (CH_3), 30.4 (CH_2), 20.9 (CH_3), 17.9 (CH_3); MS (ESI) 307 $[M]^+$.

Preparation of nasal microparticle formulation of zolpidem

The calcium carbonate microparticles were prepared by method described in ref. [23]. Zolpidem tartrate salt was incorporated into the microparticles by the adsorption technique.[24] Briefly, 15 mg of calcium carbonate microparticles were dispersed in 1 ml of zolpidem tartrate solution in water (1 mg/ml) and the suspension was mixed for 1 h on a shaker. The polyelectrolyte shell was deposited on the surface of the microparticles by electrostatic adsorption technique (Layer-by-layer method).[23,24] Namely, the particles were dispersed in 1 ml of DEAE-D solution (2 mg/ml, 0.15 M NaCl) and incubated for 15 min on the shaker. Afterwards, the particles were separated from the mixture by centrifugation and washed with water for three times. Formation of HA layer was performed using the same procedure, where 1 ml of HA solution (2 mg/ml, 0.15 M NaCl) was added to the particles followed by shaking for 15 min and washing using centrifugation/resuspension procedures. In the case of simultaneous adsorption of the polyelectrolyte and zolpidem, 0.5 ml of polyelectrolyte solution in 0.5 M NaCl and 0.5 ml of zolpidem tartrate solution (1 mg/ml) were added to the microparticles on each step of the layer deposition. After mixing for 15 min on the shaker, the microparticles were collected by centrifugation and washed with water. Loading efficiency of the microcontainers was determined by spectrophotometry calculating the difference of optical density of the initial zolpidem solution and supernatant solutions at 295 nm. To estimate the loading capacity of the calcium carbonate microparticles in weight percentage (wt %), the loading amounts of zolpidem compound (mg) were normalized by the weight of dry calcium carbonate microparticles (mg). The encapsulation efficiency of each microcontainer was calculated as follows:

$$\text{Encapsulation efficiency, wt\%} = \frac{\text{Total drug content, mg} - \text{Drug content in the supernatant, mg}}{\text{Total mass of microparticles, mg}} \times 100\%.$$

Preparation of sodium alginate film

The cross-linked polymer film was prepared as described in ref. [25]. Shortly, 2 wt% aqueous sodium alginate was poured onto a cellulose filter (Millipore) saturated with 2 wt% $CaCl_2$ solution and covered by the same filter for 15 min. The cross-linked film was washed with saline for three times.

In vitro release of zolpidem from microcontainers

Release in saline solution

The release of zolpidem was studied *in vitro* by incubation of drug-loaded calcium carbonate microparticles without shell and with polymer coating consist of two polymer bilayers (HA and DEAE-D) in saline solution. Namely, 15 mg of zolpidem-loaded microcontainers were dispersed in 10 ml of saline. At fixed intervals (10, 20, 30, 60 min) the dispersion medium was withdrawn and replaced with fresh dissolution medium. To construct the release versus time profiles and compare two proposed types of the

microcontainers (quantify the released amount of the drug), calcium carbonate microparticles were sedimented after the certain period of time and the zolpidem content in the supernatant was analysed. Drug release was quantified using a calibration curve of zolpidem. The results were expressed as a percentage of the drug released as shown below:

$$\text{Drug released}\,\% = \frac{\text{Amount of drug released at time } t, \text{ mg}}{\text{Total amount of encapsulated drug, mg}} \times 100\%.$$

Release in sodium alginate films

Sodium alginate film was placed into a Petri dish with 100 μl of saline solution slightly covering the film, and 15 mg of the microcontainers were distributed on its surface. After certain time intervals, the microcontainers were withdrawn from the film surface. To determine the zolpidem release amount, the film was placed in 5 ml of saline for 3 days and solution was analysed by spectrophotometry method as described above.

Nasal administration of zolpidem-loaded microcontainers to mice

The organization and implementation of animal experiments were carried out in accordance with the order of the Ministry of Health of Russia No. 199 dated 1 April 2016 "On approval of the rules of proper laboratory practice". Animals were kept in accordance with SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the device, equipment and maintenance of experimental biological clinics (vivariums)" of 29 August 2014 No. 51. The experiments were approved by the Commission on Biomedical Ethics of the Federal State Budgetary Institution "Research Zakusov Institute of Pharmacology" (Protocol No. 5 of 11 July 2017).

To determine the effect of zolpidem-loaded microcontainers administration by the intranasal route *in vivo* experiments was carried out applying the open field test. The subjects were male BALB/c mice (n = 50, 20–25 g). The mice were obtained from the Branch of Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry RAS in Pushchino. All mice were housed at a vivarium at the Zakusov Institute of Pharmacology in standard conditions with free access to water and food ad libitum up to 17 per cage. They were allowed to acclimate 7 days after arrival on standard diets and normal 12-h day/night regimen. The animals were divided into three groups. The saline was administered to the first (control) group, zolpidem dissolved in saline (second group) and suspension of zolpidem-loaded microcontainers in saline (third group) were administered in a dose of 3 mg/kg to the animals. The drugs were administered nasally 2 h before the study. The estimation of zolpidem anxiolytic activity was performed using the open field test.[26] The open field apparatus consists of a circular open field 65 cm in diameter surrounded by a white plastic wall 35 cm high. The surface is divided by black lines into 18 compartments plus one central and 13 holes 1.5 cm in diameter in the floor. Before experiment, the mice were kept in a darker chamber compare to the experimental field. In the beginning of each test, every mouse was introduced to the same peripheral square of the arena and was allowed to explore the arena freely for 3 min. The experiments were conducted in a sound-attenuated room with 40–50 lux light intensity in the centre of the apparatus. Recordings of entries into central and peripheral areas, rearing and climbing were made. A global activity score was composed of the scores sum for each sectors entering. The anxiolytic-like effect was calculated on the base of increased time in the central zone of the apparatus, total locomotors activity and number of rearings. The values obtained for the control and test groups were processed statistically using the program Statistica 6.0 ("Stat Soft", Tulsa, OK, USA).

Results and Discussion

Synthesis of zolpidem

Synthesis of zolpidem was achieved in five steps (Figure 1), including: (1) copper(I) chloride promoted cyclization of imine **1**, obtained from toluene aldehyde and 5-methyl-2-aminopyridine, with ethyl propiolate **2** towards imidazo [1,2-a]pyridine **3**, (2) further saponification of CO_2Et-group by potassium hydroxide results in salt **4**, (3) acidification of the former led to free acid **5**, (4) final treatment of the **5** with phosphorus pentachloride, followed by (5) in situ amidation of instable acyl chloride **6** with dimethyl amine leads to product **7**. The total yield of isolated product 7 was 19%. This synthetic approach can be considered as an adapted for scientific laboratories an excellent alternative to employed in chemical industry methods of zolpidem synthesis, relied on heterocyclizations of 2-aminopyridines with α,β-unsaturated carbonyl.[27–30]

Encapsulation of zolpidem

The amount of zolpidem incorporated in calcium carbonate by adsorption process was 5.7 wt%. To determine the influence of a polyelectrolyte shell formed on the surface of calcium carbonate microparticles loaded with zolpidem on the entrapment efficacy and release of the drug, two polysaccharides – HA and DEAE-D – were deposited on the microparticle surface by electrostatic adsorption. Zeta-

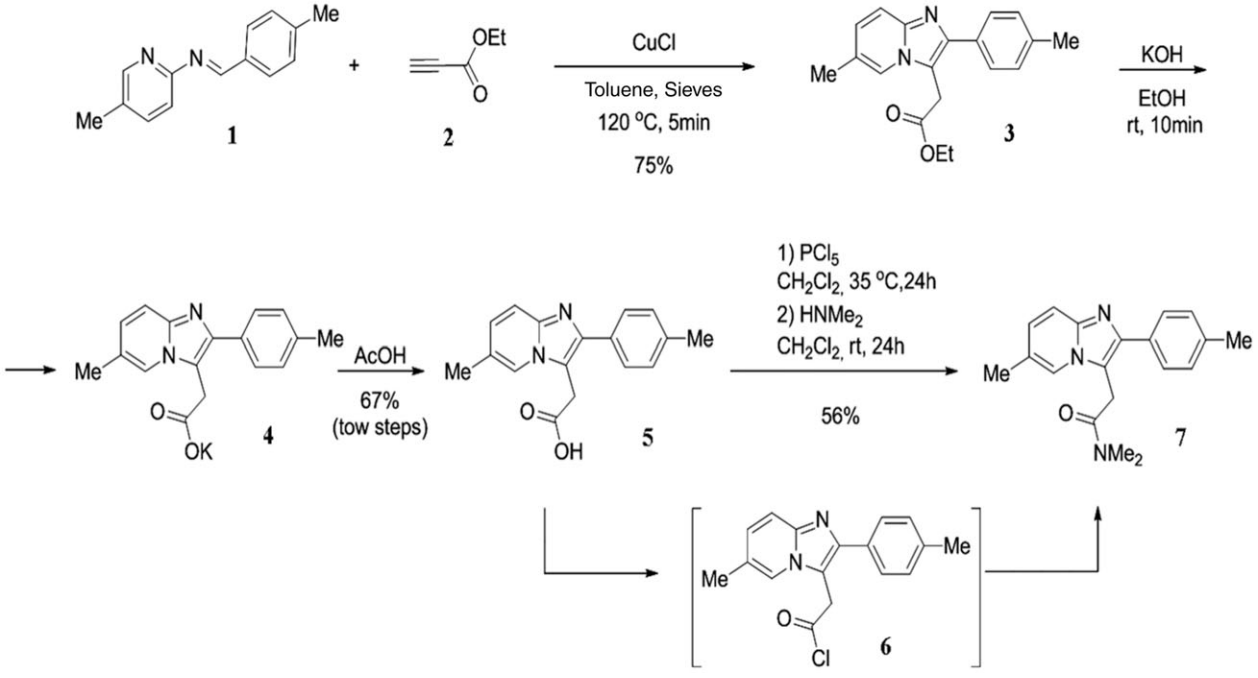

Figure 1 Scheme of zolpidem synthesis.

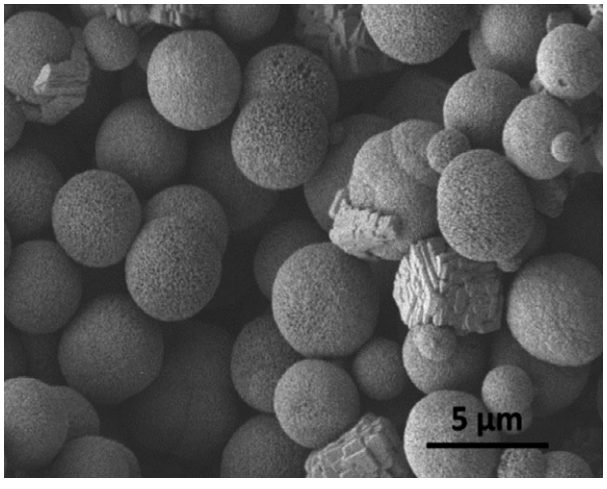

Figure 2 SEM-image of zolpidem-loaded $CaCO_3$ microparticles with (hyaluronic acid/diethylaminoethyl-dextran)$_2$ shell.

potential measurements indicate recharge of the microparticle surface after each polyelectrolyte layer deposition proving formation of the polyelectrolyte shell of the microcontainers. The microscopic image of the microcontainers with polyelectrolyte shell revealed their spherical shape and developed surface (Figure 2). The mean size of such microcontainers is 3500 ± 700 nm (PDI 0.28) (Figure S1), which is optimal for the administration through the nasal route, where particles are deposited on the nasal mucosa and reach the actual site of action.[31,32]

We found that the shell formation causes desorption of the drug from the microcontainers leading to the encapsulation efficiency decrease. One polyelectrolyte layer adsorption leads to 3 wt % loss of the encapsulated drug amount, two layers reduce encapsulation amount to 1.2 wt % (Figure 3). Finally, significant loss of the drug takes place by increasing the number of polyelectrolyte layers deposited on the microcontainers. To overcome this problem, we performed simultaneous adsorption of the polyelectrolytes and zolpidem. This method significantly improved the microcontainers loading capacity, which linearly increased with the increase in number of layers and amounted to 13.4 wt% for 4 polyelectrolyte layers (Figure 3).

Release in saline solution

The percentage of zolpidem released from the microcontainers was plotted as a function of time in Figure 4. Cumulative release of zolpidem tartrate was not total within 60 min, the released amount reached only about 50% from the encapsulated amount. The initial burst effect is common to all microspheres[33] and may have functional use in providing an initial dose during drug delivery, minimizing any lag period. It is more likely to be due to the presence of zolpidem on the surface of the microparticles

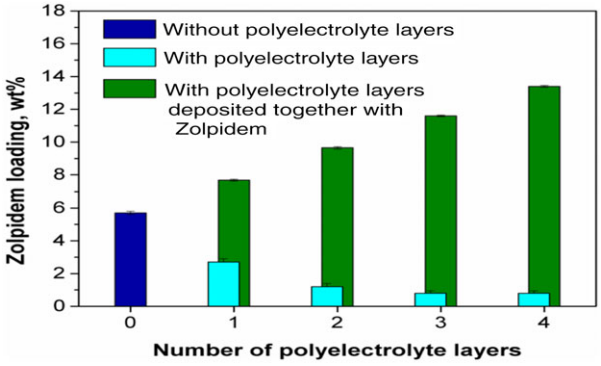

Figure 3 Loading capacity of zolpidem into $CaCO_3$ microparticles corresponding to the different entrapment procedures. The polyelectrolyte layer deposition on the microparticle surface results in zolpidem desorption from the microcontainers. Simultaneous adsorption of the polyelectrolytes and zolpidem leads to the increase in the microcontainer loading efficacy.

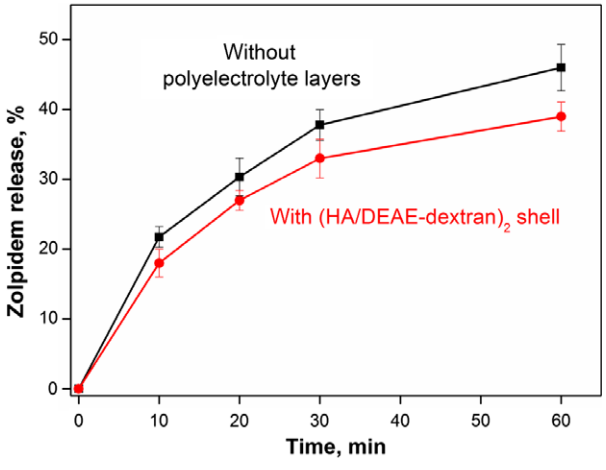

Figure 4 Release profile of zolpidem from the microcontainers into saline solution.

and its pores. This phase is followed by a controlled terminal phase.

An immersion of the microparticles covered with HA/DEAE-D in saline solution could lead to hydrophilic polymer swelling and formation of a gel diffusion layer that may hinder the outwards transport of the drug from the microcontainers, hence producing a controlled release effect. It can be seen that four polyelectrolyte layers deposited on the zolpidem-loaded microparticles have a minor effect on the release, particularly in its initial stages. Figure 3 demonstrates no significant slowdown of the zolpidem release from the microcontainers due to the polyelectrolyte layers deposition. Despite this, the

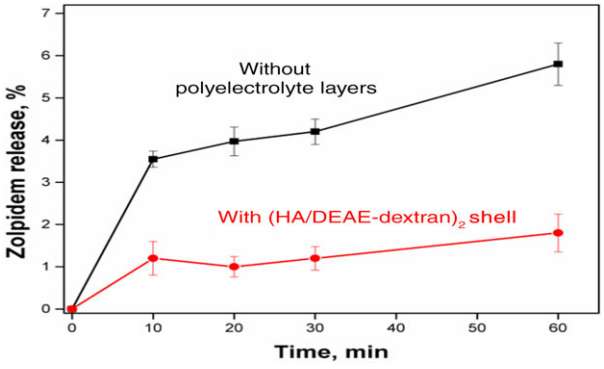

Figure 5 Cumulative zolpidem release from the microcontainers into the sodium alginate film.

formation of the polymer shell imparts mucoadhesive properties to the obtained system.

The mucoadhesive properties of the HA/DEAE-D complex may promote the residence time prolongation of the proposed microcontainers on the nasal mucosa, improving the bioavailability of the drug.[34] The numerous hydrophilic functional groups (such as carboxyl and hydroxyl groups) could form hydrogen bonds with mucus glycoprotein during the process of mucoadhesion.[35] HA molecules establish intimate contact with the mucus membrane by swelling and adsorbing the water from the mucus layer. In short, modification of the microcontainer surface with polymer coating allows improving the adhesive properties without attenuating drug release. There is also opinion that mucoadhesive microspheres could increase the drug transport by opening the tight junction of Caco-2 cells.[36]

Release in the model of nasal mucosa

We applied sodium alginate film as an elementary planar model of nasal mucosa. The release of zolpidem from two types of the microcontainers was studied: without polyelectrolyte shell and covered with four polyelectrolyte layers. It was observed that about 6% of the encapsulated drug amount was released from the uncovered microparticles in 60 min (Figure 5). Zolpidem diffusion into alginate gel found to be significantly slower compare to the release in saline. The difference in the release profile between two types of the microcontainers and methods of the release investigations is about 5%. The effect of multiple polyelectrolyte shell remains the same regardless the release conditions.

We observed a relatively slow release on a planar surface of the alginate film in a model experiment that can be explained by the lack of shaking conditions compare to first release experiment in eppendorf tubes. Forced by shaking, zolpidem desorption from the microcontainers is about 10

times higher, but these conditions poorly simulate the real *in vivo* situation. At the same time, the nasal cavity offers other conditions (differs in humidity, pH level, enzymes), suggesting a higher release of the drug. Especially enzymes can have strong effect on the release of therapeutic *in vivo*. Additionally, supernatant replacement in the case of release in saline maintains the sink conditions for zolpidem and probably enhances the amount of the desorbed drug in comparison with desorption to the film, where saturation takes place and diffusion stops.

We suggest that a permanent long-term diffusion of the drug will take place after reaching the nasal mucosa by the microcontainers, which apparently will significantly enhance the amount of the desorbed drug in comparison with the model film.

Intranasal delivery of zolpidem to the animals

Efficiency of the proposed microcontainers for intranasal delivery of zolpidem was evaluated by *in vivo* experiments using the open field test. We studied the anxiolytic effect of pure zolpidem and zolpidem-loaded microcontainers (zolpidem-loaded calcium carbonate microparticles coated with the 5-layer $(HA/DEAE\text{-}D)_2/HA$ polyelectrolyte shell) on mice.

The open field test is one of the most popular animal model of anxiety-like behaviour investigations.[37] During study of the mice behaviour, zolpidem solution (3 mg/kg) caused an increase in horizontal activity from 37.93 ± 3.41 for the control group animals to 54.00 ± 5.59 ($P < 0.05$) and the number of exits to the centre from 0.87 ± 0.24 to 4.29 ± 0.63 ($P < 0.01$). Administration of the zolpidem-loaded microcontainers in the same dose (3 mg/kg) leaded to the increase in horizontal activity from 37.93 ± 3.41 to 56.38 ± 3.61 ($P < 0.01$), vertical activity from 2.07 ± 0.82 to 8.33 ± 1.22 ($P < 0.01$) and the number of exits to the centre from 0.87 ± 0.24 to 4.46 ± 0.54 ($P < 0.01$) compare to the control group. The vertical activity (rearings) parameter was grown from 3.07 ± 0.90 for the free drug to 8.33 ± 1.22 ($P < 0.01$) for the zolpidem-loaded microcontainers (Figure 6).

Results demonstrate a classical anxiolytic activity of zolpidem in dose of 3 mg/kg (the main parameters of anxiety reduction in the open field test – horizontal activity and the number of exits to the central sector). Zolpidem-loaded microcontainers demonstrate a similar anxiolytic activity to a free drug and even exceed it by one of the parameters. This was expressed by a statistically significant increase in vertical activity (rearings).

Thus, it was shown that zolpidem has a pronounced anxiolytic effect on the behaviour of the animals in the open field test. Nasal administration of zolpidem in the form of the microcontainers improves the drug availability. The enhancement of the drug effect in the microcontainers could be attributed to residence time increase in the zolpidem-loaded microcontainers in the nasal cavity due to mucoadhesive properties of the microcontainer shell. The polymer shell facilitates prolonged and increased absorption of the zolpidem, while the free drug is removed from the nasal epithelium by mucociliary clearance in shorter period.

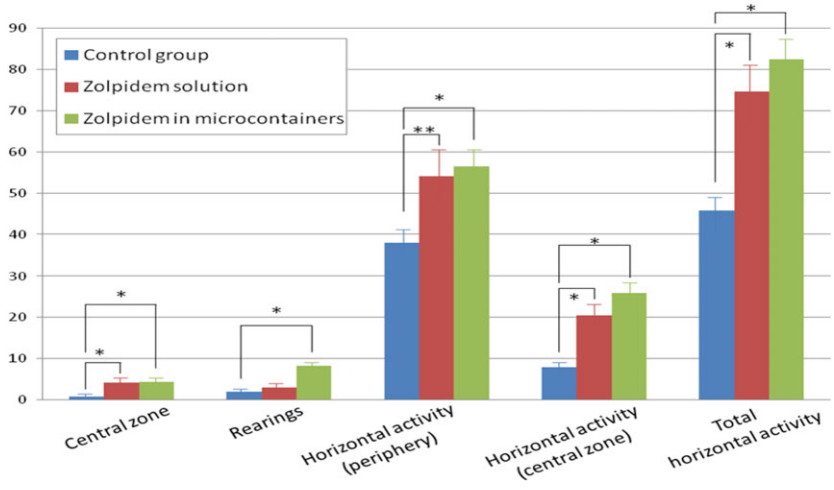

Figure 6 The results of the open field *in vivo* test for zolpidem-loaded containers. *Statistically significant difference ($P < 0.01$) from the control group; **Statistically significant difference ($P < 0.05$) from the control group; P is the statistical significance of the results according to the Kruskal–Wallis criterion.

Conclusions

The microcontainers based on calcium carbonate microparticles were developed for the encapsulation of anxiolytic drug zolpidem. The polyelectrolyte shell formed simultaneously with zolpidem increases the loading efficacy of the microcontainers and prolongs the release in saline and in the model of nasal mucosa. Rapid mucociliary clearance of intranasally administered drugs is often a key factor in deterioration of the bioavailability of the therapeutic agents. The use of mucoadhesive polymer coating provides a potential strategy for improving retention of drugs within the nasal cavity, and thereby, improves the resultant bioavailability. The results of the *in vivo* experiments demonstrate the high efficiency of the microcontainers based on calcium carbonate particles with mucoadhesive polyelectrolyte shell for the delivery of zolpidem upon intranasal administration.

Declarations

Acknowledgements

This work was performed using the equipment of the Shared Research Center FSRC "Crystallography and Photonics" RAS and supported by the Federal Agency of Scientific Organizations (Agreement No 007-ГЗ/Ч3363/26) in part of fabrication and characterization of the microcontainers and by National Research Center "Kurchatov Institute" and the Russian Foundation for Basic Research and Moscow city Government (research project № 15-33-70032 «mol_a_mos») in the part of zolpidem synthesis and anxiolytic activity study. We gratefully acknowledge Vladimir Artemov for obtaining scanning microscopy images of the samples.

References

1. Scullin MK, Bliwise DL. Sleep, cognition, and normal aging: integrating a half century of multidisciplinary research. *Perspect Psychol Sci* 2015; 10: 97–137.
2. Slavich GM, Irwin MR. From stress to inflammation and major depressive disorder: a social signal transduction theory of depression. *Psychol Bull* 2014; 140: 774–815.
3. Doghramji K. The epidemiology and diagnosis of insomnia. *Am J Manag Care* 2006; 12(8 Suppl): S214–S220.
4. Roth T, Drake C. Evolution of insomnia: current status and future direction. *Sleep Med* 2004; 5(Suppl 1): S23–S30.
5. Al-Dhubiab BE. In vitro and in vivo evaluation of nano-based films for buccal delivery of zolpidem. *Braz Oral Res* 2016; 28: e126.
6. Griebel G *et al.* Limited anxiolytic-like effects of non-benzodiazepine hypnotics in rodents. *J Psychopharmacol* 1998; 12: 356–365.
7. Pagot R *et al.* Comparison of the efficiacy and tolerability of zolpidem 20 mg and triazolam 0.5 mg in anxious or depressed insomniac patients. *Curr Ther Res* 1993; 53: 88–97.
8. Trapani G *et al.* Encapsulation and release of the hypnotic agent zolpidem from biodegradable polymer microparticles containing hydroxypropyl-beta-cyclodextrin. *Int J Pharm* 2003; 268: 47–57.
9. Villiers MM *et al.* Introduction to nanocoatings produced by layer-by-layer (LbL) self-assembly. *Adv Drug Deliv Rev* 2011; 63: 701–715.
10. Delcea M *et al.* Stimuli-responsive LbL capsules and nanoshells for drug delivery. *Adv Drug Deliv Rev* 2011; 63: 730–747.
11. Antipina M *et al.* Remote control over guidance and release properties of composite polyelectrolyte based capsules. *Adv Drug Deliv Rev* 2011; 63: 716–729.
12. Bukreeva TV *et al.* Calcium carbonate and titanium dioxide particles as a basis for container fabrication for brain delivery of compounds. *Dokl Phys Chem* 2011; 440: 165–167.
13. Borodina TN *et al.* Calcium carbonate-based mucoadhesive microcontainers for intranasal delivery of drugs bypassing the blood–brain barrier. *Bionanoscience* 2016; 6: 261–268.
14. Ishikawa F *et al.* Insoluble powder formulation as an effective nasal drug delivery system. *Pharm Res* 2002; 19: 1097–1104.
15. Wang Y *et al.* Zolpidem mucoadhesive formulations for intranasal delivery: characterization, in vitro permeability, pharmacokinetics, and nasal ciliotoxicity in rats. *J Pharm Sci* 2016; 105: 2840–2847.
16. Armarego WLF, Perrin DD. *Purification of Laboratory Chemicals*, 4th edn. Oxford, Boston: Butterworth-Heinemann, 1997.
17. Lei X. *et al.* Method for preparing compound zolpidem 2016. Patent CN 103360387 A.
18. Belyakov PA *et al.* Mechanistic insight into organic and catalytic reactions by joint studies using mass spectrometry and NMR spectroscopy. *Mendeleev Commun* 2010; 20: 125–131.
19. Oakdale JS *et al.* Ruthenium-catalyzed cycloadditions of 1-haloalkynes with nitrile oxides and organic azides: synthesis of 4-haloisoxazoles and 5-halotriazoles. *Chemistry* 2014; 20: 11101–11110.
20. Chernyak N, Gevorgyan V. General and efficient copper-catalyzed three-component coupling reaction towards imidazoheterocycles: one-pot synthesis of alpidem and zolpidem. *Angew Chemie Int Ed* 2010; 49: 2743–2746.
21. Nair DK *et al.* Synthesis of imidazopyridines from the morita-baylis-hillman acetates of nitroalkenes and convenient access to alpidem and zolpidem. *Org Lett* 2012; 14: 4580–4583.

22. Rassokhina V *et al.* Copper(II)-mediated aerobic synthesis of imidazo [1,2-a]pyridines via cascade aminome thylation/cycloisomerization of alkynes. *J Org Chem* 2015; 80: 11212–11218.
23. Volodkin DV *et al.* Matrix polyelectrolyte microcapsules: new system for macromolecule encapsulation. *Langmuir* 2004; 20: 3398–3406.
24. Sukhorukov GB *et al.* Porous calcium carbonate microparticles as templates for encapsulation of bioactive compounds. *J Mater Chem* 2004; 14: 2073–2081.
25. Borodina T *et al.* Vitamin E microspheres embedded within a biocompatible film for planar delivery. *Adv Eng Mater* 2011; 13: B123–B130.
26. Pellow S *et al.* Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 1985; 14: 149–167.
27. Kaplan J-P, George P. US4382938 A Imidazo[1,2-a] pyridine derivatives and their application as pharmaceuticals 1983.
28. Sridharan R *et al.* Glenmark Pharmaceuticals Limited, Indian Pat. Appl. 2006; 20060728.
29. Chinnapillai R. *et al.* Life Sciences Limited. PCT Int. Appl. 2009; 2009007995
30. Labriola R. Procedimiento Para Preparar n,n,6- trimetil-2- (4-metilfenil)-imidazo-[1,2-a] -piridina-3- acetamida y sus sales WO 2000008021 2000.
31. Hatch TF. Distribution and deposition of inhaled particles in respiratory tract. *Bacteriol Rev* 1961; 25: 237–240.
32. Stuart BO. Deposition of inhaled aerosols. *Arch Intern Med* 1973; 131: 60–63.
33. Volodkin D. CaCO3 templated microbeads and-capsules for bioapplications. *Adv Colloid Interface Sci* 2014; 207: 306–324.
34. Lim S *et al.* In vivo and in vitro characterization of novel microparticulates based on hyaluronan and chitosan hydroglutamate. *AAPS PharmSciTech* 2001; 2: e20.
35. Arora P *et al.* Permeability issues in nasal drug delivery. *Drug Discov Today* 2002; 7: 967–975.
36. Harikarnpakdee S *et al.* Spray-dried mucoadhesive microspheres: preparation and transport through nasal cell monolayer. *AAPS PharmSciTech* 2006; 7: E79–E88.
37. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol* 2003; 463: 3–33.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1. The size distribution of the microcontainers.