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Buspirone transdermal administration for menopausal syndromes, *in vitro* and in animal model studies

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ABSTRACT

Menopausal syndromes can seriously disturb the quality of women's life. In this work, we have investigated transdermal administration of buspirone, a 5-HT_{1A} receptor agonist, for treatment of the major menopausal syndrome, hot flushes. To the best of our knowledge, this is the first time buspirone has been proposed for the treatment of hot flushes. We designed a buspirone transdermal system containing the drug in an ethosomal carrier. Pharmacokinetic data in rats following transdermal administration indicate that buspirone was present in plasma for 12 h, reaching a C_{max} value of 120.07 ± 86.97 ng/ml after 2 h. A F_{rel} value of 0.89 was estimated for transdermal vs. oral buspirone. The effect of transdermal buspirone on hot flushes was evaluated in ovariectomized rats by monitoring tail skin temperature changes. Temperature alleviation (1.6 ± 0.7 °C) to normal values was observed 3 h post-buspirone administration with the effect lasting for at least 3 h. Histological examination of the skin at the application site indicated that transdermal ethosomal buspirone is safe. The significant findings presented here encourage further studies with ethosomal buspirone transdermal system for treatment of menopausal syndromes.

1. Introduction

Published work suggests that a strong association exists between anxiety and vasomotor symptoms in menopausal women, with the most anxious women reporting higher frequencies of hot flushes (Freeman et al., 2005; Woods and Mitchell, 2005; Hunter and Rendall, 2007; Li et al., 2008; Simon and Reape, 2009). The brain serotonergic system has a functional role in the development of both anxiety and hot flushes (Barnes and Sharp, 1999; Berendsen, 2000; Gross et al., 2002; Stearns et al., 2002; Strzelczyk et al., 2004) where the serotonin (5-hydroxytryptamine-1A [5-HT_{1A}]) receptor plays a key role in the anxiety etiology. Interestingly, 5-HT_{1A} and 5-HT_{2A} receptor subtypes are also believed to be closely associated with body temperature control.

Buspirone hydrochloride (BH), a currently used anxiolytic, exerts most of its clinical effects by modulating the serotonergic system and acts as a full agonist at the presynaptic 5-HT_{1A} receptor in the raphe nuclei, where it inhibits the firing of serotonin neurons as well as the synthesis and use of serotonin. Postsynaptically, BH is a partial agonist at 5-HT_{1A} receptors, resulting in increased 5-HT_{1A} activity (Tunnieliff, 1991; Ohlsen and Pilowsky, 2005).

The goal of this work was to study the efficiency of BH transdermal administration in animal models for anxiety and hot flushes. One hypothesis tested in the present work was that BH, by affecting the 5-HT_{1A} receptor in the brain, could be efficient in treating the two menopausal syndromes, anxiety and hot flushes.

Buspirone oral administration has a number of disadvantages. Although it is rapidly absorbed in the GI, this drug undergoes extensive first-pass metabolism and has a very short elimination half-life. Therefore, efficient oral treatment requires a multiple daily dose regimen (Mahmood and Sahajwalla, 1999; Sakr and Andheria, 2001). It is assumed that by transdermal administration these drawbacks could be avoided. On the other hand, BH is a hydrophilic cationic molecule that does not possess enough skin permeability to enable therapeutic blood concentrations. For this reason, we designed and tested a BH transdermal system using ethosomes as the enhancing permeation carrier. Ethosomes are vesicular carriers designed by us for enhanced delivery of active agents into the deep layers of the skin and across the skin. The main components of the ethosomal system are phospholipids, high concentrations of ethanol and water (Touitou, 1998; Touitou et al., 2000, 2008; Ainbinder and Touitou, 2005).

2. Materials and methods

2.1. Materials

Buspirone hydrochloride (BH) was a gift of Unipharm, Israel. Other materials used were: carbomer (Carbopol® 940, Noveon,

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USA), phospholipid (Phospholipon® 90G, Phospholipid GMbH, Germany), rhodamine 6G and sodium chloride (Sigma–Aldrich, Israel), potassium dihydrogen phosphate (Merck, Germany), ethanol (Gadot, Israel), acetonitrile, methanol (J.T. Baker, Holland) and tocopheryl acetate (Tamar Ltd., Israel).

2.2. Preparation of BH vesicular systems

BH ethosomal systems containing 3% drug, 2.5% phospholipid, 0.2% tocopheryl acetate and 38% (w/w) ethanol in carbomer gel were used in this work. The systems were prepared as follows: the aqueous solution of the drug was added slowly with continuous mixing at 700 rpm (Heidolph, Germany) to the ethanolic solution of phospholipid and tocopheryl acetate. The mixture was then added to a 0.7% carbomer gel to obtain the final ethosomal system (15, 16). For fluorescent studies, 0.01% rhodamine 6G (R6G) was incorporated in the above BH ethosomal system. BH carbomer aqueous gel was used as a control system.

2.3. Physical characterization of BH vesicular systems

2.3.1. Visualization of BH vesicles by transmission electron microscopy (TEM) and cryo-scanning electron microscopy (cryo-SEM)

BH ethosomes were visualized by TEM using a Philips TEM CM 12 electron microscope (Philips, The Netherlands). Samples were negatively stained on a carbon-coated copper grid with ammonium molybdate. The specimen was viewed under the microscope at 58k-fold enlargements. Cryo-SEM visualization was performed following application on electron microscopy copper grid and flash freezing in slushy nitrogen under vacuum. The sample was coated with gold by a Polaron E5100 sputter coater (Polaron, UK) and visualized under Philips 505 scanning electron microscope (Philips, The Netherlands) at an accelerating voltage of 53 kV.

2.3.2. BH surface tension activity determination

The surface tension values of BH in aqueous solutions were measured at room temperature by the ring method using the Du Nouy ring tensiometer (Fisher, USA).

2.3.3. Measurements of vesicular size distribution and zeta potential

The size distribution, polydispersity index (PdI) and zeta potential of ethosomes were determined by dynamic light scattering (DLS) using a computerized Malvern Zetamaster-nano, ZEN 3600 inspection system (Malvern Instruments, UK). Vesicles size distribution was measured in two sets of triplicates in an automatic mode of 60 s each at a medium stable count rate.

2.3.4. Differential scanning calorimetry (DSC) measurements

The phase transition temperature, T_m, of vesicular lipids was measured by using a Mettler DSC 30, connected to a computer with a Mettler Toledo Star Software System (Germany). For measurements aluminum sample pans (aluminum crucibles 40 μl with pin, ME-27331, Mettler Toledo, Germany) were used. The thermal analysis was performed in the temperature range of −70 to 70 °C, at a heating rate of 10 °C/min, under a constant nitrogen stream. T_m values given are the temperatures recorded at the peak minimum.

2.3.5. Determination of entrapment efficiency

The entrapment efficiency of ethosomal vesicles was determined by the ultracentrifugation method. Samples were centrifuged in a TL-100 ultracentrifuge (Beckman, USA) equipped with TLA-45 rotor at 40,000 rpm for 3 h at 4 °C. Accurately weighted quantities of the supernatant were diluted with appropriate medium and injected to HPLC for drug quantification. The entrapment efficiency was calculated using the equation: $[(T - S)/T] \times 100$, where T is the total drug quantity both in the supernatant and sediment, and S is the quantity detected in the supernatant.

2.4. Evaluation of R6G skin penetration by CLSM

The depth of R6G skin penetration from BH vesicular system vs. aqueous system was determined by means of confocal laser scanning microscopy (CLSM). Systems (100 mg) were applied nonocclusively for 12 h on full-thickness defrosted porcine ear skin (Lahav, Israel) in Franz diffusion cells (PermeGear, Bethlehem, PA). The receiver medium was 0.01 M PBS (pH 7.4). At the end of the experiment, the excess of the preparation was carefully wiped from the skin surface and the treated area of the skin cut out. The whole skin was optically scanned at 5 μm increments through the z-axis with an Olympus FV 300 (Japan) CLS microscope attached to an universal Olympus fluorescence microscope having air plan 10 × 0.40 NA objective lens. Optical excitation was carried out with a 543-nm argon laser-beam and fluorescence emission was detected above 560 nm. Relative fluorescence intensity of the probe (arbitrary units) in the whole skin was further assessed using an Image Pro-Plus Software. Pinhole size, electron gain, neutral density filters and background level were set up at the beginning of the experiment and were kept constant during fluorescence measurements of probe from all of the systems investigated by this method.

2.5. BH skin permeation studies in vitro

The permeation of BH from the vesicular vs. aqueous systems was measured through the full-thickness porcine ear skin in experiments carried out for 24 h at 37 °C in Franz diffusion cells. One hundred mg of BH ethosomal system or BH aqueous system were applied nonocclusively on a 1.77-cm^2 diffusion area. Samples (200 μl) were withdrawn from the receiver (3:7, v/v, ethanol:water) at various time intervals and were replaced by the same volume of fresh receiver fluid.

BH was quantified by reverse phase HPLC, using a Merck-Hitachi D-7000 apparatus equipped with an L-7400 variable UV detector, L-7300 column oven, L-7200 auto-sampler, L-7100 pump, and an HSM computerized analysis program. The assay was carried out at 240 nm, using a Zorbax Eclipse XDB C18, 5 μm and 4.6 mm × 150 mm column kept at 30 °C, with a mobile phase composed of acetonitrile:phosphate buffer 0.01 M pH 7.2 (40:60, v/v) at 1 ml/min. Cumulative corrections and calculation of permeation kinetics parameters were performed using our Transderm program (Touitou and Watenfeld, 1987).

2.6. Determination of BH plasma concentration in rats

The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center for animal welfare approved all animal study protocols. The Hebrew University is an AAALAC International accredited institute. Animal experiments were conducted in full compliance with the approved protocols.

Animals were housed under normal 12-h light/dark cycle and a temperature of 21–22 °C, with food and water freely available. Ten male Wistar rats (Harlan, Israel), 300–330 g in weight, were randomly divided into two equal groups. A day before the experiment the back area of 5 cm × 6 cm was shaved using an electrical clipper (Oster, USA). BH was applied on the skin at a total dose of 15 mg/kg as follows: the formulation containing 30 mg/g BH was applied nonocclusively on 16 cm^2 of the dorsal skin of each of the five rats. The animals were restricted by hands for 5 min after application and then the rats were placed in individual cages. The other five rats received an oral dose of 3 mg/kg BH aqueous solution by

gavage needle. BH aqueous solution was prepared by dissolving BH in water in concentration of 2 mg/ml. Blood samples of 500 μl were collected, at several pre-defined intervals after dosing (1, 2, 4, 6, 8, 11 and 24 h), by 250 μl heparinized glass capillary tubes into 1.5 ml vials. Following centrifugation at 4000 rpm for 10 min, plasma was separated and then frozen immediately at −20 °C until assayed. Before detection, the samples were thawed and then 20 μl of methanol, 300 μl of acetonitrile and 80 μl NaCl saturated solution were added. The mixture was centrifuged for 15 min at 6000 rpm. 50 μl of supernatant were injected into HPLC. Plasma standard curve was prepared from whole blood samples collected from intact rats. Stock solution of BH (in methanol) was added to yield final plasma concentrations ranging from 15 to 750 ng/ml. The samples were then prepared for analysis as described above. Analysis of BH was carried out using a Nucleosil C18, 125 mm × 4 mm, 5 μm column with a mobile phase of acetonitrile: phosphate buffer 0.01 M pH 5.8 (40:60, v/v) at 1 ml/min.

2.7. Pharmacokinetic parameters from in vivo experiments

The AUC_{0-24} was calculated by the trapezoidal rule for the time interval 0 to the last measurable point, 24 h. The peak plasma concentration, C_{max}, and time to reach the maximum drug plasma concentration, t_{max}, were obtained from the concentration–time plot. The relative bioavailability (F_{rel}) was calculated from the relationship $F_{rel} = (D_{po} \times AUC_{TS})/(D_{TS} \times AUC_{po})$, where 'po' is oral and 'TS' is transdermal administration.

2.8. Measurement of the effect of BH administration on elevated tail skin temperature (TST) in hot flushes rat model

In this study we used the estrogen deficiency-associated thermoregulatory dysfunction rodent model, and measured changes in tail skin temperature of ovariectomized rats (OVX) (Kobayashi et al., 2000; Berendsen et al., 2001; Opas et al., 2004). OVX rats exhibit elevated TST during the active (dark) phase of the day.

Bilateral ovariectomies or sham-surgeries (which left their ovaries intact) were performed to female Sprague–Dawley rats (Harlan, Israel), 250–330 g in weight, followed by 2 weeks of recovery period. Prior to the experiment the back area of 5 cm × 6 cm was shaved using an electrical clipper. One day before treatment, baseline measurements were performed to OVX and sham rats. TST of the sham-operated rats was considered as the normal tail skin temperature. On the day of experiment, nine OVX rats were divided into two groups of four and five animals. The first group received 5 mg/kg BH aqueous solution subcutaneously. In the second group of OVX animals, BH vesicular system at a dose of 15 mg/kg was applied nonocclusively on 16 cm^2 of the back skin. The animals were restricted by hands for 5 min after application and then placed in individual cages. Thermal measurements were conducted during 5 min on animal kept in a flat bottom restrain. Thermocouple skin sensor probe SST-1 (Physitemp Instruments Inc, USA) was affixed to the dorsal surface of their tails approximately 1 cm from the base of the tail. TST measurements were performed through the use of Thermalert TH-5 (Physitemp Instruments Inc., USA). All measurements were performed during the dark period.

2.9. Measurement of anxiolytic effect in elevated T-maze anxiety rat model

The effect of the vesicular BH system was measured on the elevated T-maze anxiety model introduced by Graeff and co-workers (Graeff et al., 1993, 1998; Pinheiro et al., 2007). The T-maze apparatus (in-house made) consists of three elevated arms of equal dimension (50 cm × 12 cm) elevated 50 cm from the floor, one enclosed by lateral walls (40 cm high) and two open. When placed at the end of the enclosed arm, the rat does not see the open arms until he pokes his head beyond the walls of the closed arm. Rats have an innate fear of height and openness, thus to be on an open arm is an aversive experience. Repeated placement inside the enclosed arm to explore the maze allows the animal to learn inhibitory avoidance. Twenty-five male Wistar rats (Harlan, Israel), weighing 250–350 g, were randomly assigned to five groups. A day before the experiment the back area of 5 cm × 6 cm was shaved using an electrical clipper (Oster, USA). The first group was used as control without treatment ($n = 8$). The second group received intraperitoneal injection (IP) of 1 mg/kg diazepam ($n = 4$) with the measurements performed 25 min after administration. In this work diazepam, a conventional benzodiazepine anxiolytic was used to test the model validity. Consistent with previously reported results on this model, diazepam significantly decreased the inhibitory avoidance 2 in comparison to the control untreated group (Graeff et al., 1993, 1998; Duzzioni et al., 2008). In the third ($n = 4$) and fourth group ($n = 5$) of animals, 15 mg/kg of BH vesicular system was applied nonocclusively on 16 cm^2 of the back skin. For these transdermal groups, the measurements were started 4 h (third group) and 12 h (fourth group) after transdermal application. The BH oral solution treated group ($n = 4$), received aqueous BH solution of 2 mg/ml by gavage needle, at a dose of 3 mg/kg and the anxiolytic effect was assessed 12 h after administration. Each rat was placed at the end of the enclosed arm of the T-maze and the time to leave this arm with the four paws was recorded (baseline). The same measurement was repeated in two subsequent trials (avoidance 1 and avoidance 2) at 30-s intervals. Cutoff time was 600 s.

2.10. Skin histological examination

Histological examination of the skin sections at the site of the treatment was performed to evaluate any change in the state of the skin (thickness of the horny layer, cyst and bubble formation). Skin samples from animals that underwent the pharmacokinetic experiment were examined following routine fixation with 4% formaldehyde, paraffin block processing and staining with haematoxylin–eosin. Microscopic evaluation was performed by a blinded assessor.

2.11. Data analysis

All results are expressed as mean values ± standard deviations. Statistical analyses were performed using one-way ANOVA at a significance level set at $P < 0.05$ or less. When a statistical analysis was performed for only two groups, an unpaired two-tailed t-test was used.

3. Results

3.1. Characterization of buspirone transdermal system

BH ethosomal system was characterized for structure, mean size distribution, zeta potential, softness and entrapment efficiency.

TEM and SEM observations show that BH ethosomes are unilamellar vesicles (Fig. 1A) with a closed spherical shape (Fig. 1B). The unilamellarity of ethosomes could be due to the effect of the drug, a surface active molecule, on the vesicular structure. We found that increasing the concentration of BH from 0.05 to 2.4 mM caused a reduction in the surface tension of water of 11 mN/m, to a plateau value of 58 mN/m.

The mean size distribution of empty vesicles was 232.8 ± 51.6 nm (PdI = 0.62). A small decrease in the vesicle size was observed following addition of 30 mg/g BH to the system (194.8 ± 98.6 nm, PdI = 0.75). While ethosomes without drug

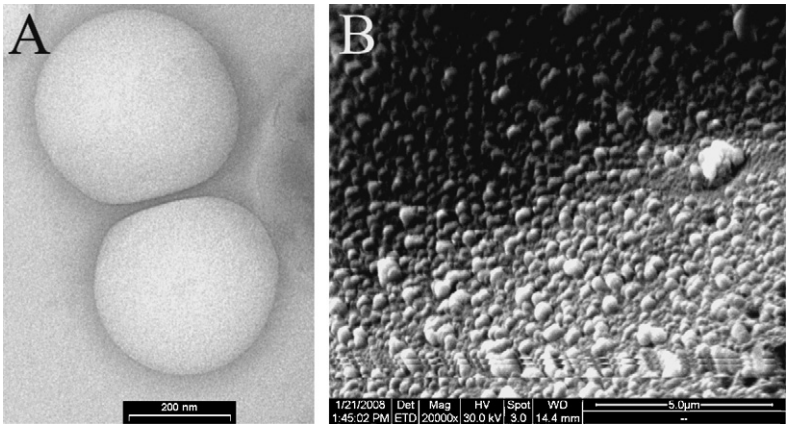


Fig. 1. Electron micrographs of buspirone ethosomal system obtained by (A) transmission electron microscopy (58,000×) and (B) scanning electron microscopy (20,000×).

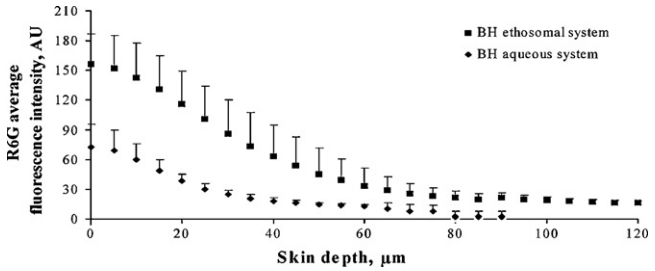


Fig. 2. R6G fluorescence intensity profiles as a function of skin depth showing the penetration of R6G into the porcine skin during a 12-h experiment. Depth of skin penetration and fluorescence intensity were assessed by CLSM and analyzed by the Image Pro-Plus Software. Mean ± S.D.; $P < 0.01$.

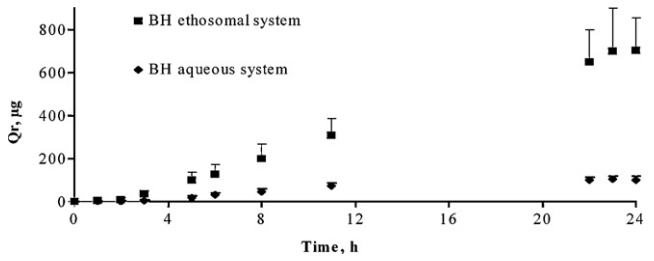


Fig. 3. Cumulative amount (Qr) of buspirone (BH) that permeated across porcine skin during a 24-h experiment from ethosomal system ($n = 4$) and aqueous system ($n = 4$). Mean ± S.D.; $P < 0.05$ for 2 and 3 h; $P < 0.01$ for 5, 6, 8 and 11 h; $P < 0.001$ for 22, 23 and 24 h time points.

exhibited a negative charge of −8.8 mV, the addition of 30 mg/g BH increased the vesicle charge to +7.16 mV.

The melting temperatures of the lipids in the vesicular systems as measured by DSC were −46.83 and −44.32 °C for empty ethosomes and BH ethosomes, respectively.

The drug entrapment efficiency of ethosomal vesicles was found to be 75.3 ± 1.5%.

3.2. Delivery into/across the skin

3.2.1. Depth of skin penetration

R6G, being a cationic hydrophilic molecule with a similar molecular weight, was used in these experiments as a model probe for BH.

The profiles of R6G penetration vs. skin depth following 12 h application on porcine ear skin show that the average fluorescence intensity in various skin depths was significantly higher ($P < 0.01$) when the probe was delivered by the ethosomal system in comparison to the aqueous one (Fig. 2). The delivery from ethosomes resulted in an increase in both depth of penetration (120 μm vs. 80 μm) and fluorescence intensity in the skin.

3.2.2. Buspirone permeation across the skin

The time-dependent skin permeation profiles of BH showed a classic permeation behavior, with a lag phase followed by steady-state flux (Fig. 3). It could be noticed from these plots that ethosomes significantly increased the amount of BH that permeated over time across the skin relative to the aqueous system ($P < 0.01$).

A flux of 18.77 ± 4.4 μg/cm^2 h drug, was calculated from the linear portion of the plot, for BH delivered from the vesicular system. This value is significantly higher than the 4.74 ± 0.6 μg/cm^2 h BH flux measured from the aqueous system. The lag time values, although without statistical significance, were shorter for the BH vesicular system than for the aqueous system (117 ± 37 min vs. 146 ± 38 min, respectively).

3.3. Buspirone bioavailability in rats: transdermal vs. oral administration

Plasma drug concentration profiles following transdermal administration of 15 mg/kg BH from a formulation containing 30 mg/g drug and a single dose of 3 mg/kg oral administration of aqueous drug solution are presented in Fig. 4.

BH was detected in rat plasma 60 min post-transdermal or oral administration. Transdermal BH did not achieve a statistically significant different C_{max} value from the oral administration, 120.07 ± 86.97 and 93.44 ± 76.46 ng/ml, respectively. The t_{max} values were 2 h for transdermal administration and 1 h for the

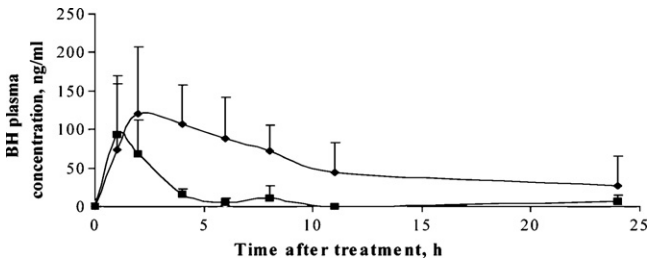


Fig. 4. BH rat plasma concentration profiles following (♦) transdermal ethosomal system application ($n = 5$) and (■) oral administration ($n = 5$). Mean ± S.D.; $P < 0.05$ for 4, 6, 8, and 12 h time points.

Table 1
Pharmacokinetic parameters after BH transdermal and oral single dose administration to rats.

System	Drug dose (mg/kg)	t_{max} (h)	C_{max} (ng/ml)	AUC_{0-24} (ng h/ml)	F_{rel} transdermal vs. oral
BH transdermal ethosomal system	15	1	120.07 ± 86.97	1349 ± 847*	0.89
BH oral solution	3	2	93.44 ± 76.46	303 ± 216	

BH: buspirone hydrochloride; F_{rel}: relative bioavailability. Mean ± S.D.; $n = 5$.
* $P < 0.05$ significant difference from oral BH (unpaired t-test).

Table 2
Effect of BH administration on inhibitory avoidance in elevated T-maze rat model.

System	Drug dose (mg/kg)	Baseline (s)	Withdrawal latency Inhibitory avoidance		n
			Avoidance 1 (s)	Avoidance 2 (s)	
Untreated control	–	30 ± 33	289 ± 185	573 ± 51	8
BH transdermal ethosomal system					
4 h after application	15	26 ± 20	165 ± 115	316 ± 130*	4
12 h after application	15	50 ± 27	68 ± 44	205 ± 118**	5
BH oral solution					
12 h after administration	3	33 ± 21	263 ± 132	576 ± 42	4

BH: buspirone hydrochloride. Mean ± S.D.
* $P < 0.01$, significant difference from untreated control (ANOVA with Tukey–Kramer multiple comparisons test).
** $P < 0.001$, significant difference from untreated control (ANOVA with Tukey–Kramer multiple comparisons test).

oral aqueous solution (Table 1). Noteworthy, significantly higher plasma concentrations ($P < 0.05$) were measured at $t = 4$, 6, 8, and 12 h, for the transdermal BH.

A relative bioavailability (F_{rel}) value of 0.89 was estimated for transdermal vs. oral BH.

3.4. Pharmacodynamic effects

3.4.1. Effect in hot flushes rat model

During this study, we observed that intact rats (sham-operated) exhibited lower tail skin temperature (TST) values in comparison to ovariectomized (OVX) rats during the active period. Measured 2 weeks after operation, the mean TST value in OVX rats was significantly higher ($P < 0.01$) than the value in sham rats (29.74 ± 0.5 °C vs. 28.4 ± 0.6 °C, respectively) with a ΔTST (°C) of 1.34 °C. In a further study the TST values of untreated OVX rats were used as control. Subcutaneous injection of BH to OVX rats caused a relative rapid reduction of TST, achieving normal values 2 h after injection. This effect was maintained for about 3 h followed by a second wave of TST raised values (Fig. 5A). The above findings suggest that BH could be efficient in hot flushes treatment and this effect should be further investigated.

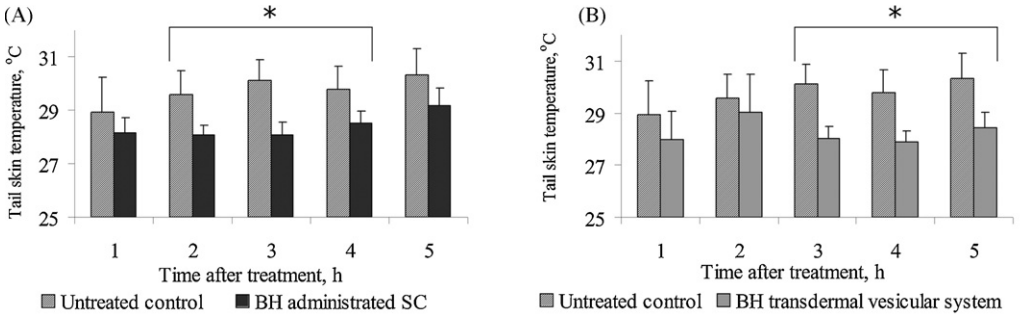
A significant TST decrease was observed 3 h post-transdermal BH administration. The tail temperature remained low for at least 3 h up to the end of the experiment (Fig. 5B).

3.4.2. Anxiolytic effect in T-maze anxiety rat model

BH transdermal system impaired avoidance acquisition 4 and 12 h after 15 mg/kg dose administration (Table 2). The avoidance latency at the second trial was significantly shorter in BH transdermal groups than in the control group ($P < 0.01$). It is worth mentioning that BH did not alter the inhibitory avoidance 12 h after oral administration of the drug.

3.5. Skin histological examination

Histological examination of the skin sections at the site of the transdermal treatment did not reveal any change in the state of



	Dose mg/kg	Onset h	Duration h	Mean ΔTST °C	Max ΔTST °C
BH administrated SC	5	2	3	1.47 ± 0.5	2.1
BH transdermal ethosomal system	15	3	>3	1.6 ± 0.7	2.1

Fig. 5. Effect of buspirone (BH) following: (A) subcutaneous ($n = 4$) and (B) transdermal administration ($n = 5$) on elevated TST in OVX rats. TST are measured in the active phase 1, 2, 3, 4 and 5 h after treatment (TST: tail skin temperature; OVX: ovariectomized). Mean ± S.D.; * $P < 0.05$ compared to untreated OVX group.

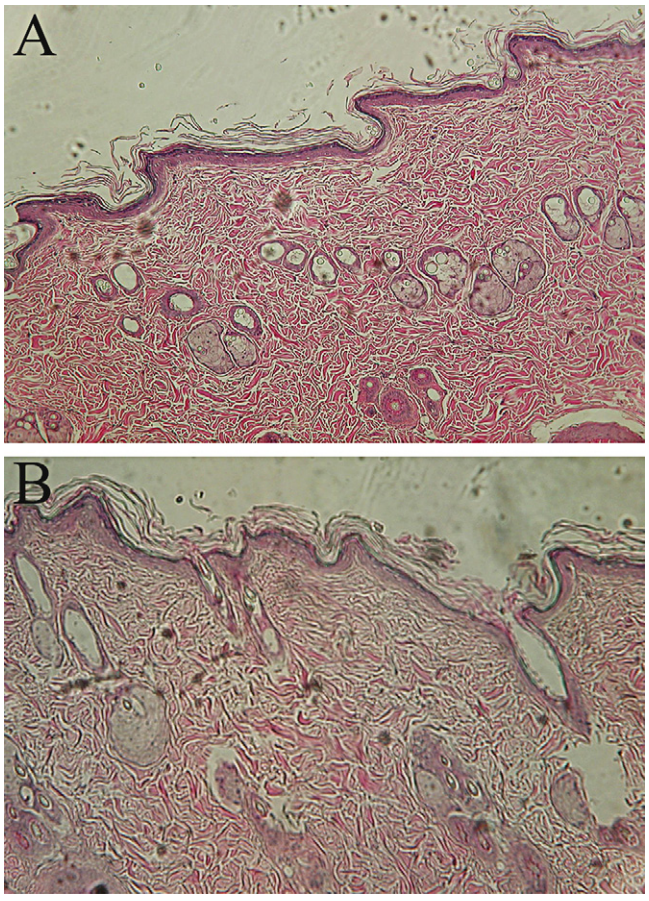


Fig. 6. Histological rat skin sections, stained with hematoxilin–eosin, obtained from animals treated for 24 h with (A) buspirone ethosomal transdermal system and (B) untreated animals.

the skin vs. normal untreated rat skin (Fig. 6). The transdermal ethosomal system containing buspirone, applied for 24 h, did not cause either infiltration of inflammatory cells or cyst and bubble formation in the skin. No histological evidence of inflammation was found. Thickness and appearance of the horny layer were found to be not changed in comparison to the normal untreated rat skin.

4. Discussion

The search for alternative treatments of hot flushes is in the forefront today in light of findings of a WHI (Women's Health Initiative) study showing increased risks of breast cancer and cardiovascular diseases in menopausal women taking hormone replacement therapy (Morimoto et al., 2002; Pradhan et al., 2002). In this respect there are numerous reports on the use of selective serotonin reuptake inhibitors (SSRI) and serotonin-norepinephrine reuptake inhibitors (SNRI) as alternative treatments for menopausal syndromes (Loprinzi et al., 2002; Evans et al., 2005; Soares et al., 2006; Shen and Stearns, 2009).

In the present work we have investigated the efficiency of BH for treatment of hot flushes and anxiety. BH is an anxiolytic drug used in the treatment of generalized anxiety disorder (GAD) and anxiety caused by alcohol craving or smoking cessation (Apter and Allen, 1999). Some studies indicate that BH could be efficient for long-term treatment of GAD and major depression and have a prophylactic effect in migraines associated with anxiety disorder (Rickels et al., 1991; Schweizer et al., 1998; Lee et al., 2005; Mitte et al., 2005). This drug has advantages over other anxiolytics since it lacks anticonvulsant or muscle relaxant properties, does not impair psychomotor function, does not cause sedatation or physical dependence and does not potentiate the effects of alcohol. It appears to lack detectable deleterious effects on cognition when administered acutely at clinically meaningful doses (Apter and Allen, 1999; Bourin and Lambert, 2002; Chamberlain et al., 2007).

BH regulates the 5-HT_{1A} serotonin receptor function in the brain. This receptor has been reported to play a key role in the hot flushes etiology, the major climacteric symptom that seriously disrupts daily life. Keeping these processes in mind, we hypothesized here that BH could be effective in the treatment of the two most prevalent menopausal syndromes, anxiety and hot flushes.

To prove the above, we first tested BH on ovariectomized rat (OVX), an animal model of hot flushes. Administered subcutaneously, BH caused a decrease in tail skin temperature of OVX rats already 1 h after administration and achieved a significant alleviation in the thermoregulatory dysfunction of the rats during the next 2 h. Consistent with the reported drug elimination half-life in rats, the total duration of the effect was about 3 h. To the best of our knowledge, we are here reporting for the first time the efficacy of buspirone, an anxiolytic agent, on hot flushes treatment (Touitou, 2009).

Based on these findings and aiming to overcome the extensive first-pass metabolism and multiple daily dose regimens associated with the oral administration, we have designed a BH transdermal ethosomal system. The main characteristics of the ethosomal system contributing to the mechanism of drug permeation enhancement across the skin are the bilayers fluidity of the soft phospholipid vesicle in conjunction with the presence of high concentrations of ethanol in the system (Touitou, 1998; Touitou et al., 2000).

The designed BH ethosomal system contains cationic unilamellar soft vesicles of about 200 nm with entrapment efficiency of 75%. Although a number of properties of ethosomes such as mean size distribution and entrapment efficiency were measured in this work, based on the proposed mechanism of permeation enhancement by the soft ethosome, it is important to keep in mind that these properties do not contribute to the enhancement ability of the system. The unilamellarity of the BH ethosomes could be explained by the surface-activity of the drug which interacts with phospholipid bilayers.

Investigating the enhancing effect of BH ethosomal system on the skin delivery of R6G, we have found that the probe penetrates the skin to a much greater depth than from the aqueous control system. Additionally, permeation studies show that buspirone flux across skin was about four times higher from the vesicular composition relative to the aqueous system. It was then estimated that BH delivered from an ethosomal system having an effective area of 70 cm^2 could achieve efficient anxiolytic blood levels in humans (Touitou, 1990; Brunton et al., 2005). In previous studies investigating BH permeation through mouse skin, enhanced permeation was achieved by using terpenes chemical enhancers and iontophoresis. The combination of these two modalities showed a synergistic effect (Al-Khalili et al., 2003).

Results of our pharmacokinetic study show that, when administered transdermally, the drug was present in rat plasma for a much longer period compared to the oral administration, 12 h vs. 4 h, respectively. By providing a non-fluctuated and continuous delivery of BH into the bloodstream, transdermal administration may offer sustained efficacy with reduced side effects, thus leading to improved patient compliance. A relative bioavailability of 0.89 was estimated for transdermal vs. oral administration.

The next step of our work was to study the pharmacodynamic responses induced by transdermal administration of BH from the vesicular carrier. The application of BH ethosomal system on the skin of ovariectomized rats caused a decrease in the elevated tail

temperature 3 h after administration, which continued for a total period of 6 h until the end of our experiment. We then studied the anxiolytic effect of the BH transdermal system in comparison with oral drug administration. This was evaluated using an elevated T-maze anxiety model. Based on the previous PK study, we chose to test the system's efficacy at two time points. We found a significant decrease of avoidance latency 4 and 12 h after BH transdermal system application in comparison to the untreated group. Consistent with data obtained in the PK experiment which showed that 12 h after oral administration the drug quantities in the plasma were negligible, the inhibitory avoidance latency was affected only in rats that received the transdermal BH system and not in oral treated animals ($P < 0.001$). These results are supported by the PK profile found for this system.

Finally, the safety of the transdermal system is of great importance. Taking into consideration that many enhancers, which interfere with the barrier function of the skin, are irritants, we tested the skin after the BH transdermal administration. Histological observations of the skin at the site of treatment show that the application of BH ethosomes did not induce changes in the skin structure and in the thickness of the horny layer. No infiltration of inflammatory cells to the skin was found. Such results indicate that the BH ethosomal transdermal system is safe and does not irritate the skin. These outcomes are in line with our long experience with ethosomes in *in vitro* and in clinical use (Touitou, 1998; Horwitz et al., 1999; Touitou et al., 2000, 2008; Ainbinder and Touitou, 2005; Paolino et al., 2005).

5. Conclusions

The findings of this work suggest that buspirone, an axiolytic drug, could be efficient in hot flushes treatment and therefore should be further investigated. Anxiety, an emotional disorder, is strongly associated with menopausal hot flushes that disturb women's lives. Treatment of these most prevalent symptoms in menopause could strongly benefit this patient population. The findings of our work show that the ethosomal buspirone transdermal system could be considered as a promising delivery system for the treatment of menopausal syndromes. This system showed enhanced skin permeation *in vitro*, good bioavailability and efficient pharmacodynamic responses in animals.

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