Steam bath, vibration, and thermal ablation administrations augment the release of tramadol HCl from transdermal patch and enhance the plasma concentration in rats

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ABSTRACT: Recently, transdermal drug delivery has become popular due to their numerous advantages. They offer non-invasive application and eliminate the first-pass metabolism. The skin membrane is sensitive to heat and vibration that these applications enhance the skin permeability resulting in increased bioavailability. This study aims to determine the effect of steam bath (STB), vibration (VIB) and thermal ablation (THAB) on systemic absorption of tramadol HCl and compare all applications with each other. After preparing of tramadol HCl patches, in vitro release tests followed by in vivo animal experiments were conducted. The patches were applied to 32 Wistar albino rats divided into four groups: No potential trigger effect (NPTE), STB, VIB, and THAB. STB, VIB and THAB were applied by purchased devices. One hour later, the patches were removed and plasma concentration of tramadol HCl was measured by UV-Vis spectrophotometry at 271 nm. When compared to the control group, calculated plasma tramadol HCl µg/mL concentration increased significantly in STB, VIB and THAB ($p < 0.001$). Finally, as trigger effects, steam bath, vibration and thermal ablation (42°C) dramatically increased the absorption amount of tramadol HCl (42.1%, 37.2 % and 43.8 %, respectively). The percentage release of tramadol HCl increased significantly in investigation groups when compared to NPTE ($p < 0.001$). In conclusion, controlled heat and vibration applications are effective in the enhancement of transdermal drug absorption.

KEYWORDS: Transdermal patch; Steam bath; Vibration; Thermal ablation; Tramadol HCl.

1. INTRODUCTION

Tramadol hydrochloride (HCl) is a currently approved synthetic hydrophilic analgesic drug used for the treatment of moderate to severe pain [1]. It centrally acts as an opiate agonist, via binding to the µ-opioid receptor, and weak inhibition of norepinephrine and serotonin uptake [2]. However, the tramadol HCl has many side effects such as nausea, vomiting, sweating, constipation, drowsiness. In addition, tramadol HCl led to uncontrollable nervous tremors. The risk of developing addiction or resistance is low when compared with other analgesics [3]. Tramadol has a half-life of approximately two and a half hours and is metabolized by the CYP2D6 enzyme in the liver. It is commercially available in many dosage forms in Turkey in various formulations such as tablets, capsule, solution, injection and oral drop. However, there is no transdermal formulation that is advantageous for increasing systemic effect with a higher bioavailability and reducing toxic and side effects in humans. The stratum corneum is the outermost layer of the skin which is produced by keratinocytes embedded in a rich content lipid layer (fatty acids, cholesterol, and ceramide), it has a natural physical barrier function to prevent the subcutaneous parts from external damage, infection, and dehydration [4]. To enhance the penetration of drugs from the skin, chemical enhancers such as ketones, fatty acids/fatty acid esters, sulfoxides, and azone, and/or physical enhancers such as electrophoresis, iontophoresis, and thermal ablation are preferred [5]. These methods apply high energy to the skin for the increasing penetration of active ingredients from the skin [6]. Especially, thermal techniques usually heat the skin surface, and destroy the stratum corneum (destructing the lipid structure) through disturbing the keratin framework to produce micron-sized pores, thereby enhancing skin permeability, especially of hydrophilic macromolecular drugs like tramadol HCl [7]. The other most preferred systems are transdermal delivery systems. The most

challenging part of these systems is passing the stratum corneum layer of the skin. These systems facilitate the penetration of drugs through the skin into the systemic circulation. In 1979, Food and Drug Administration (FDA) agency in the United States approved the first transdermal system, scopolamine patch, against motion sickness [8]. After that, a range of transdermal patches for the treatment of several illnesses was approved and released to the market. Transdermal drug delivery is accepted as a non-invasive route of administration and offers remarkable advantages. The transdermal route provides remarkable enhancement of bioavailability by eliminating the first-pass metabolism of drugs in the liver [9]. Since no loss by hepatic degradation occurs, the drug can be administered in lower doses, decreasing the eventual side effects of the drug. Moreover, it provides longer periods of drug release, eliminating plasma fluctuations [10, 11]. In addition, in transdermal therapies, patients' compliance is more because it is painless, convenient, therapeutically more effective, well-tolerated, esthetical, easy-to-handle, and cost-effective. Furthermore, a controlled analgesia delivery system ameliorates functional status, ability to work, and quality of life of patients [12, 13]. Because of these advantages, several TDDSs are available in different drug therapies, currently. On the other hand, these systems need extra enhancers to provide increased permeability resulting in higher bioavailability of drugs [14, 15].

In the light of these information, the objective of current study is to demonstrate and compare the effect of steam bath, vibration and thermal ablation as trigger effects on the concentration of tramadol HCl released from the patch and entered to the plasma. Second aim of this study is to highlight the impact of trigger effects on percentage release of the percentage release amount of tramadol HCl.

2. RESULTS

2.1. Preparation of calibration curve and regression analysis for in vitro experiments

Tramadol HCl was analyzed by using spectrophotometric methods in buffer (pH 5.5) media. The range of wavelength and the range of concentration were chosen in order to optimise the conditions. Beer's law was obeyed in the linearity range of 4-40 µg/mL, the wavelength of 271 nm. (Max absorbance was found at 271 nm).

The absorbance values of tramadol HCl dissolved at concentrations of 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 µg/ml in Sorenson's phosphate buffer were measured at 271 nm and the calibration curve was plotted and the linear equation was found to be y=0.0062x+0.0013, as shown in Figure 1.
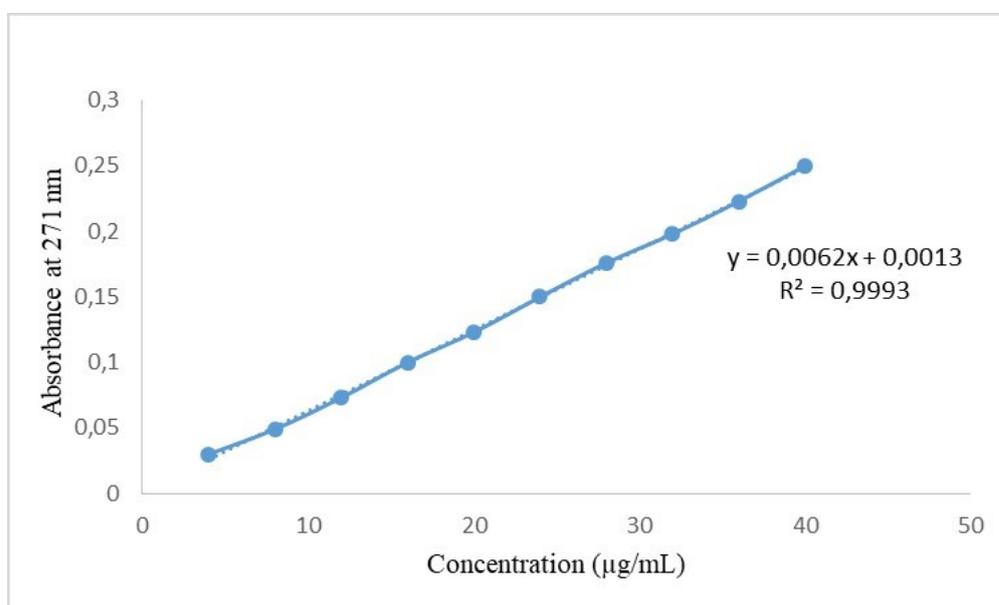

Figure 1. Calibration curve of tramadol HCl in Sorenson's phosphate buffer

The calibration curve showed a linear relationship between the concentration of tramadol HCl and their absorbance values. As the concentration increased, a proportional increase in absorbance was observed in the curve (R^2=0.9993).

2.2. In vitro drug release study

In the dissolution test, three patches, prepared with the same formulation (A, B and C), were randomly selected and then dissolution release procedures were done in triplicates with them. As a result, all three patches showed a similar release of tramadol HCl into the buffer. All patches were completely dissolved. Tramadol HCl reached its highest value in the 45-60 min. and all three patches showed similar profiles in the release study shown in Figure 2.
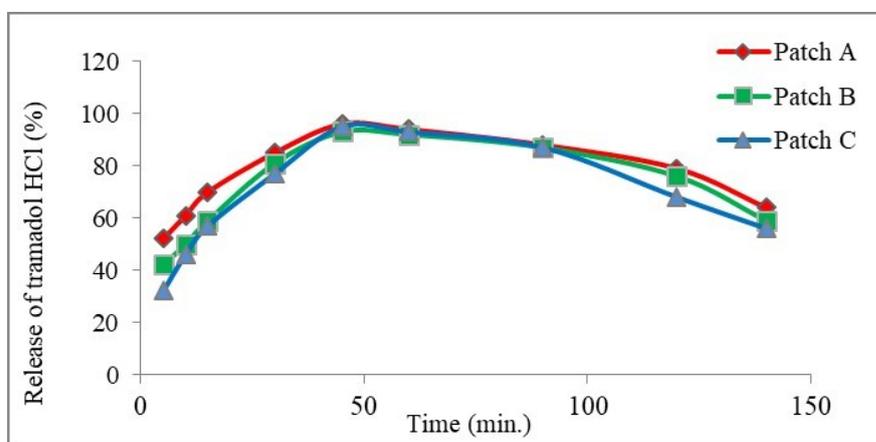

Figure 2. In vitro dissolution profiles of the three patches over time (n=3 Patch A, B, and C)

2.3. Determination of tramadol HCl concentration in rat plasma

Drawn calibration curve by using blank rat plasma showed a linear relationship between the concentration vs. the absorbance of tramadol HCl (R^2=0.9994) and the equation was found as y=0.018x-0.6604, as given in Figure 3.
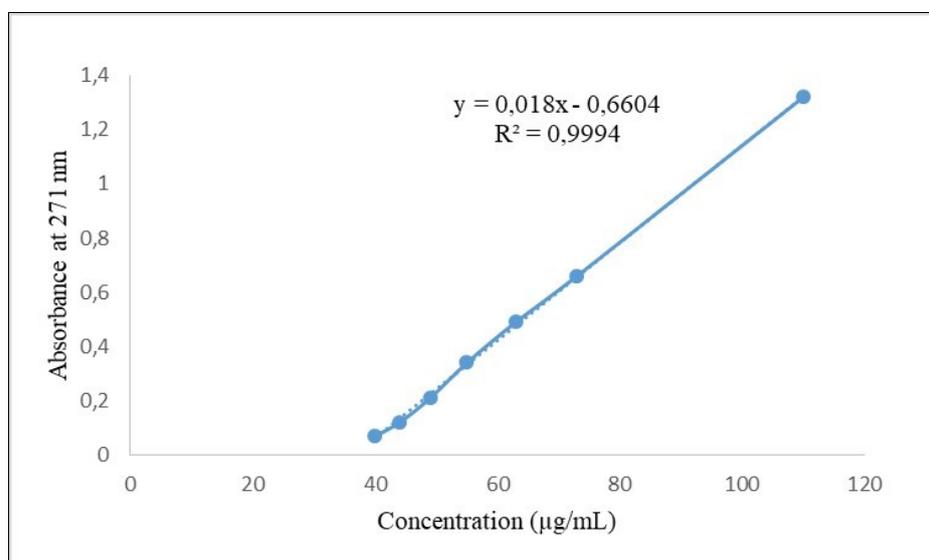

Figure 3. Calibration curve of tramadol HCl in rat plasma

Placebo rat plasma was used and the calibration curve was plotted to add tramadol to a certain amount. It was thought that methanol is a more suitable solvent for tramadol. The standard calibration curves of tramadol were constructed by plotting absorbance versus concentration in the determined concentration range with the final dilution. Developed UV spectrophotometric methods in this study are accurate, sensitive, precise and reproducible and can be directly and easily applied to the plasma. Placebo rat plasma was used and the calibration curve was plotted to add tramadol to a certain amount. It was thought that methanol is a more suitable solvent for tramadol dissolution.

Table 1. Validation results of in vitro and in vivo methods

	Phosphate buffer solution	Rat plasma
λmax (nm)	271	271
Beer's law limit (µg/mL)	4-40	4-40
Regression equation	0.0062x+0.0013	0.018x-0.6604
Slope (OD/µg/mL)	0.0062	0.018
Intercept (OD)	0.0013	0.6604
R²	0.9993	0.9994
LOD (µg/mL)	12.67636	29.91746
LOQ (µg/mL)	38.41322	90.65897

LOD: Limit of detection; LOQ: Limit of detection; R²: Linearity/Correlation coefficient; OD: Optical density

When compared to the NPTE group, tramadol HCl (µg/mL) concentration in rat plasma was increased significantly by STB, VIB and THAB administrations ($p<0.001$). The findings were presented in Table 2.

Table 2. The percentage release amount of tramadol HCl

Group	Calculated plasma concentration of tramadol HCl (µg/mL)	The amount of tramadol HCl in plasma (µg)	The percentage release amount of tramadol HCl (%)
NPTE	114.10±2.17	228.21±4.34	16.2±3.87
STB	135.56±5.52***	271.12±10.63	42.1±6.52***
VIB	132.43±4.97***	268.23±8.77	37.2±4.31***
THAB	137.25±4.49***	274.50±8.98	43.8±9.83***, $

NPTE: No potential trigger effect; STB: Steam bath; VIB: Vibration; THAB: Thermal ablation; HCl: Hydrochloride. The values were shown as Mean±SD (n=8 in each group). ***$p<0.001$ significant compared to NPTE, $$p<0.05$ signifcant compared to VIB.

Furthermore, similar to calculated plasma concentration results, all trigger effect models were statistically significant than NPTE group in term of the percentage release amount of tramadol HCl ($p<0.001$). Moreover, it was observed that there was a significant difference between VIB and THAB groups in term of the percentage release amount of tramadol HCl, 37.2%±4.31 and 43.8%±9.83, respectively ($p<0.05$). In addition, STB provided high release (42.1%±6.52) of tramadol HCl when compared to VIB (37.2%±4.31); however, the difference was not significant ($p>0.05$), as can be seen in Table 2 and Graph 4.
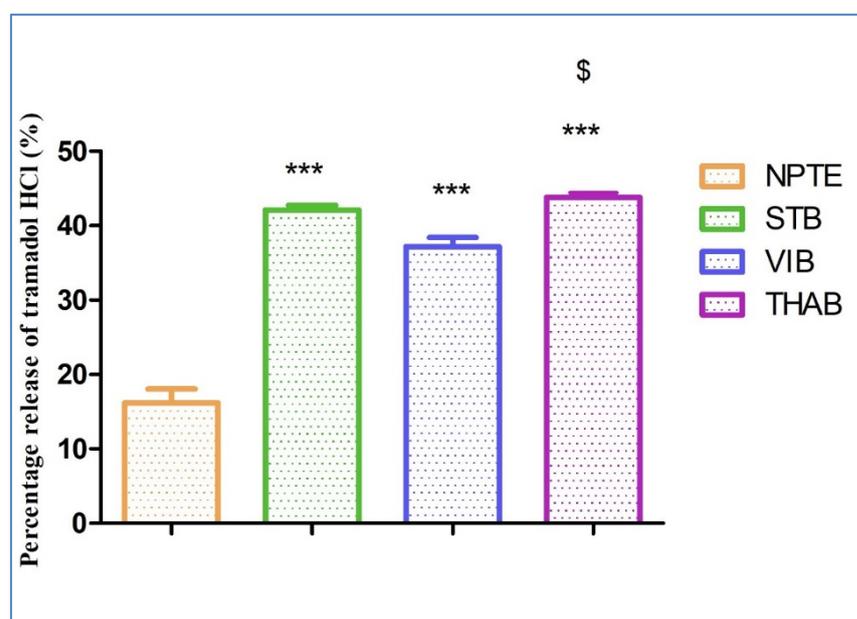

Figure 4. The percentage release amount of tramadol HCl (Statistical analysis). NPTE: No potential trigger effect; STB: Steam bath; VIB: Vibration; THAB: Thermal ablation; HCl: Hydrochloride. The values were shown as Mean±SD (n=8 in each group). ***$p<0.001$ significant compared to NPTE, $$p<0.05$ signifcant compared to VIB.

3. DISCUSSION

Tramadol hydrochloride (HCl) is, mostly preferred centrally acting opiate agonistic analgesic drug, used for the treatment of pain. It has been well known that it shows its effect via binding to the μ-opioid receptor, and weak inhibition of norepinephrine and serotonin uptake. Because it affects various receptor types and neurotransmitters, it has many side effects like nausea, vomiting, sweating, constipation, drowsiness. On the other hand, the risk of developing addiction or resistance is low when compared with other analgesics [3]. In term of pharmakinetics, half life of tramadol HCl is nearly two and a half hours and is metabolized by the CYP2D6 enzyme in the liver. Therefore, bioavailability of it decreases, as expected. In order to provide approximately complete bioavailability, transdermal formulation becomes very advantageous. It also reduces toxic and side effects in humans Patches are the most appropriate formulations to avoid first-pass metabolism and to reduce risks for side effects and addiction. Although they are loaded with low doses of drug molecules, they can show the optimal therapeutic level [9].

It is known that, TDDSs use for enhancing transdermal biologic delivery has been minimal, especially when used alone [1]. For the last four decades, especially in clinics, transdermal patches have been preferred in the transdermal delivery of small molecule anesthetics, hormones and opioids like drugs such as tramadol HCl, to improving diffusion in the intact skin pathway [16, 17]. Although TDDSs have higher bioavailabilty, to increase the penetration of drugs from the skin vibration and thermal heating methods are combined with TDDSs [5]. Improved skin perfusion following the heat applications and vibration may be mediated through different mechanisms including local and neural mechanisms such as activation of axon reflex and nitric oxide synthase (NOS) systems [18, 19, 20]. However, the exact mechanism still needs to be determined.

In the current study, both in vitro and in vivo studies were performed. Primarily, the patches were prepared by using the same formulation which included 10% PVA solution, 10% propilen glycol and 7 mg of tramadol HCl for each patches. To determine releasing time of patches, USP apparatus 5 was used. These patches were shown the similar releasing profiles that the tramadol HCl in same formulated patches (n=3) reached the maximum concentration in buffer solution at 45 minutes. Results of the in vivo studies showed, the trigger effects (STB, VIB and THAB) were administrated to experimental animals. At the end of the experimental procedures, tramadol HCl concentrations in plasma were determined. Findings demonstrated that all trigger models enhanced the permeability and the releasing amount of tramadol HCl into the rat plasma. Similarly, different studies demonstrated that absorption of some drugs embedded in patches were increased by heat application [21, 22].

Recently, various methods have been used to increase transdermal drug delivery by increasing the permeability of the skin. One of the most popular is the application of heat. There have been plenty of studies that the application of heat to the skin caused enhancing microcirculation and increasing of blood perfusion in the skin. As a result of these effects, permeability of skin increased resulting in promoting absorption of drug transdermally [23, 24]. Moreover, in a study performed with fentanyl, Shomaker et al. stated that administration of local heat with controled heat-aided drug delivery (CHADD) patch started analgesia quickly. They also reported that local heat provided earlier diagnosis and treatment of side effects by affecting the steady state concentration of fentanyl in the TDDD system [25]. Shin et al. performed a study investigating the transient heat application on in vitro skin permeation and human pharmacokinetics of three different fentanyl transdermal delivery systems, in order to evaluate in vitro and in vivo correlation of these TDDD systems. Results of study indicated that heat administration enhanced fentanyl bioavailability in in vivo study higher than those observed in vitro for all three drug products [26]. Similar to these studies, we performed with low dose (7 mg/kg) of tramadol HCl into each patch and we observed significant improvements in our results that percentage release of tramadol HCl was increased in STB, VIB, and THAB groups. In a different study, it was investigated that the effects of heat on fentanyl release and permeation through heat-separated human epidermis from a TDS. It was found that fentanyl release from TDS was significantly increased at 40°C compared with the release observed at a normal skin temperature of 32°C [27]. Furthermore, it has also been reported in another study that a local application of heat at 43°C for 60 seconds causes a significant increase in the perfusion of the skin without giving rise to pain or harm [28]. These results are consistent with our findings of current research that heat administration remarkably improved permeability of drugs transdermal drug delivery systems. These results are evidence of our results that heat enhances the permeation of the drug from the patch to the skin layers.

Moreover, another trigger effect performed in this study was vibration. There is even a patent proving that vibration increases transdermal drug delivery. Bernabei utilized a device to provide almost simultaneous vibrations and bursts of electric pulses between 40 to 60 Hz to the skin to enhance transdermal drug delivery [29]. There have been many researches in the literature on this topic. In a study investigating the in vitro and in vivo effectiveness of vibrating microneedles the on transdermal delivery of vitamin C, the microneedles

were administered at three different intensity levels (level 1, 2 and 3), five different application times (1, 3, 5, 7, and 10 minutes) and three different application powers (500, 700 and 1000g). Obtained findings of both studies exhibited that vibrating microneedles significantly increased the permeability of Vitamin C from the skin resulting in enhanced $AUC_{0-\infty}$ and C_{max} in rats [30]. Moreover, Liu et al. performed a study to investigate the effect of vibration on transdermal delivery of insulin. According to the results of the in vivo experiment on insulin absorption, it was demonstrated an enhanced transdermal delivery of insulin through vibrating micro-needles treatment. In addition, the degree of enhancement increased with vibration frequency[31]. In another study detected whether the use of vibration can change skin temperature in young women with lipodystrophy, it was observed that the application of the series of vibrations improved skin blood supply and contributed to the reduction of gynoid lipodystrophy among the participants in young subjects [32]. In a previous study conducted by Sintov et al., application of high-frequency alternating current (100 kHz) results in the formation of microchannels, while exposure to higher frequencies (100–500 kHz) caused ionic vibration within the tissue and both led to enhancement in transdermal delivery of hydrophilic drugs [33]. In a different study evaluating the benefits of various devices included vibration device on histamin-induced itch and mustard oil-induced pain, the administration of vibratory stimulus relieved the itch and pain [34]. These results are in accordance with our results that vibration application significantly increases heat, resulting in improved transdermal drug delivery.

Furthermore, another method to deliver hydrophilic and high molecular weight drugs in transdermal drug delivery systems is thermal ablation. Thermal ablation is defined as a method used to deliver drugs systemically through the skin by heating the surface of the skin, which depletes the stratum corneum selectively at that site of heating only, without damaging deeper tissues [5, 35, 36]. The main target for application of thermal devices (41 to 42 ±0.1°C) is both to increase permeability of the skin and to enhance the bioavailability of drug systemically [37]. In a review article written by Arora et al. and Parhi and Mandru, the effect of plenty of micro-scale devices on transdermal drug delivery was discussed and it was stated that local heating of the skin with thermal ablation of the cells, resulting in enhanced drug transport across the skin both in animal and in human trials [38, 37]. There have been plenty of studies about use of thermal ablation in transdermal delivery of various drugs. In a study, the possibilities of delivering antiviral protein interferon alpha-2b (INFa2b) employing thermal ablation with/without iontophoresis. Thermal ablation method enhanced the delivery of INFa2b in rats when combined with iontophoresis [39]. Lee et al. studied transdermal delivery of nicotine and the effect of heat application in this delivery. Results showed that thermal ablation devices can rapidly apply energy and hence heat to the skin, giving rise to selective removal of stratum corneum without damaging deeper tissue [5]. In a different study performed previously, 7 patients with different scars were treated with triamcinolone acetonide and 5-Fluorouracil combined thermal ablations. At the end of the study, Visual Analogue Scale (VAS) was used to measure pain relief. Finding showed remarkable mean treatment pain VAS score. Satisfaction level of patients was rated as moderate–high [40]. In another study investigating the influence of thermal administration on transdermal delivery of diclofenac, findings demonstrated that thermal ablation improved the bioavailability of diclofenac [41]. In a previous study, controlled heat was administrated to the transdermal fentanyl patch in order to determine pharmacokinetic profile of fentanyl. According to the findings, significant rapid increases in serum concentration occured and controlled heat shortened the time that needed to reach clinically important [42]. Findings of current study showed similarity with the results of previous studies that thermal ablation significantly increased permeability of skin resulting in enhanced plasma concentration of tramadol HCl.

Current study demonstrated that thermal applications (steam bath condition and thermal ablation device) and vibration like trigger effects increase release of tramadol HCl from patch by increasing the permeabilty of skin resulting in raised bioavailability of tramadol HCl in plasma. Hyperthermia applications require raising an assigned temperature to a predetermined level within a given time and maintaining that temperature, with acceptable temperature gradients, for a predetermined time interval. Based on this research, the optimum conditions, effectiveness for the use of thermal devices and STB for transdermal hydrogel formulation containing tramadol has been established. From the present knowledge, what temperature gradients within surrounding rat skin would be tolerable (it seems that 41 to 42 ±0.1°C, in rat skin) may be reasonable. Although this tolerated rate would presumably depend upon the final temperature to be achieved. The 41-42°C range has both the potential to increase tramadol HCl absorption with trigger effects (Steam bath, Vibration and Thermal Ablation) and also to improve penetration through blood circulation, approximately two times more than without trigger effect hydrogel application.

It is postulated that steam bath, vibration, and thermal ablation may alter skin permeability resulting in the increased absorption of tramadol HCl. That the amount of tramadol HCl was greater at the vibration and thermal ablation suggests those trigger effects could increase tramadol HCl delivery almost two or three times increased.

4. CONCLUSION

In conclusion, it has been well known that the stratum corneum acts as a stong barrier that limits the penetration of substances through the skin. This study compared the effect of steam bath, vibration, and thermal ablation techniques on the transdermal drug permeation whether they lead to an increase or not. In addition, this study targeted to demonstrate effect of these administrations on plasma tramadol HCl concentration. In this study, it was reported that a treatment with heat and/or vibration also efficiently increased tramadol HCl absorption from rat skin. We proposed trigger effects (steam bath, vibration and thermal ablation) with hydrogel in the present study as an effective strategy to enhance the absorption of tramadol. There are many illnesses that have been waiting for radical treatment. Current study suggested that transdermal patches combined with heat administrations or vibration will become very effective in many disorders. Transdermal drug delivery offers an attractive alternative to the conventional drug delivery methods of oral administration and injection. It is obviously said that thermal devices were really promising methods as a novel drug delivery system for analgesia treatment. Although further studies are needed, the results of the present study provide evidence for the increasing absorption of the trigger effects with in vivo studies as well as its potential for clinical applications. Finally, as trigger effects, steam bath, vibration and thermal ablation (42°C) significantly increased the absorption amount of tramadol HCl (42.1%, 32.2 % and 43.8%, respectively).

5. MATERIALS AND METHODS

5.1. Formulation of transdermal tramadol HCl single layer patch

A transdermal patch containing tramadol HCl (model drug) was formulated by using polyvinyl alcohol (PVA) as a carrier polymer, and propylene glycol (10%) as a plasticizer. 10% PVA solution was prepared by dissolving PVA in an isotonic sodium chloride solution and stirring proceeded (500 rpm) for a few more minutes. Then, 7 mg of tramadol HCl was added to each well-plate and agitated with the help of a syringe tip. The plate was placed in the oven and dried at 50°C and 10% relative humidity (RH) for approximately four hours. Finally, transparent patches with a diameter of 1 cm were obtained.

5.2. In vitro experimental study

5.2.1. Preparation of calibration curve and regression analysis

The calibration curve is necessary for the determination of the time of arrival maximum concentration of drug in rat plasma. The first step of the drawing calibration curve was to prepare the known concentration of tramadol HCl solution in Sorenson's phosphate buffer was scanned 200-500 nm by a UV-vis spectrophotometer and the maximum wavelength was determined at 345 nm. Tramadol HCl solution was prepared in Sorenson's phosphate buffer with different concentrations, which were of 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 µg/ml. The absorbance was measured by using respective concentrations. The calibration curve was drawn for the in vitro experiments. It is indicating that the concentration of tramadol HCl was obtained by knowing the absorbance value. The calibration curve of tramadol HCl was drawn with concentrations and then R^2 value was calculated.

5.2.2. In vitro drug release study

In vitro drug release studies of tramadol HCl from three transdermal patches were performed by using the paddle over disc method (USP apparatus 5). The vessels of the apparatus were filled with 250 mL of phosphate buffer at pH 5.5 and 32±0.5°C. The paddle speed was set at 50 rotations per minute (rpm). When the equilibrium was achieved, dry films of the patches (3.8 cm² surface area) were fixed into the disk assembly, then they were transferred into the vessels. Aliquots of 1 mL were taken from the release medium at regular predetermined time intervals (5, 10, 15, 30, 45, 60 and 90 minutes) and fresh dissolution medium was added after taking samples. These studies were performed under sink conditions. The samples were measured in UV-Vis spectrophotometer (Agilent) at 271 nm [43]. This study was performed as triplicates.

5.3. In vivo experiments

5.3.1. Experimental animals

Thirty-two adult male Wistar Albino rats (250-300 g; 8 week-old) were randomly assigned to one of the four groups (8 rats in each): No potential trigger effect (NPTE), Steam bath (STB), Vibration (VIB), and Thermal ablation (THAB). They were acclimatized with free access to water and regular chow and were kept on a 12 h light-dark period and maintained at a constant temperature of 22±2°C. All experimental procedures were carried out in conjunction with the National Institution of Health's Guide to Care and Use of Laboratory Animals and ethic approval of this study was obtained from the local Animal Ethics Committee (02/12/2014-425).

5.3.2. Experimental procedures in groups

After the random assignment of the rats into four groups (8 rats in each): No potential trigger effect (NPTE), Steam bath (STB), Vibration (VIB) and Thermal ablation (THAB). The dose of a model drug, tramadol HCl (Sigma Aldrich), was chosen as 7mg/kg for each patch for rats [44]. Animals (n=32) were anesthetized with a combination of ketamine-xylazine (50/10 µg/g). The back of the rats was shaved and the tramadol HCl patch was applied on this area for one hour according to the following experimental procedures. Patches were applied to animals in the NPTE group, directly for one hour. In the STB group, patches were applied to the back of the rats and the heat was applied by an experimental far infrared-ray dry sauna system (Metos, Tokyo, Japan) at 41°C for 60 minutes, with minor modifications [45]. The heat was monitored using a thermometer and was kept at the interval 41-42°C. In the VIB group, the patches were applied on the back of the rats followed by vibration was administered with the designed device only the first one minute. The patches were removed from the rats one hour later. In the THAB group, the heat was applied by designed device on the back of the rat for 40 seconds followed by patches were administrated. Designed devices were obtained from Biomedical engineering laboratory. The patches were fixed on the back of the rats by using cotton gauze and the bandage. Finally, the blood samples from animals were collected intracardiacly into heparinized tubes. The tubes were centrifuged for 15 minutes at 4500 rpm and stored at -20°C until analysis.

5.3.3. Preparation of calibration curve for in vivo experiments

The calibration curve is necessary for releasing the concentration of drug from the patch to rat plasma. The first step of drawing the calibration curve was to prepare the known concentration of tramadol HCl solution in blank rat plasma, which was scanned 200-500 nm by UV-vis spectrophotometer and the maximum wavelength was determined at 271 nm. Tramadol HCl solution was prepared in the blank rat plasma with different concentrations which were 40, 44, 49, 55, 63, 73, 110 µg/mL. The absorbances were measured by using respective concentrations. The calibration curve was drawn for the in vivo experiments. It is indicating that the dissolution profile of the tramadol HCl patch was obtained at the time of releasing the maximum drug concentration from the patch.

5.3.4. Determination of the amount of tramadol HCl in rat plasma

A spectrophotometric method was used to determine the concentration of tramadol HCl in rat plasma. Blood samples were collected at regular time intervals (5, 10, 15, 30, 45, 60, and 90 minutes). The plasma sample was obtained from each animal and mixed with Sorenson's phosphate buffer by vortex machine. The absorbance was measured at 271 nm and the R^2 value was determined by using on UV-Vis spectrophotometer (Agilent HP G1103A 8453, USA).

5.4. Validation

The Beer's law limits, regression equation, correlation coefficient, LOD, and LOQ were calculated mathematically with placed formulas below and findings were presented in Table 1.

(Eq. 1) $LOD = (3.3 \times \sigma)/S$
(Eq. 2) $LOQ = (10 \times \sigma)/S$

where the σ is the standard deviation of the response and S is the slope of the calibration curve.

The linearity can be detected by the high value of correlation coefficient. As these values demonstrated that the method is sensitive.

5.5. Statistical analysis

GraphPad Prism v 5.03 was used for the statistical analysis. Data were analyzed by One-way Analysis of Variance (One-way ANOVA), followed by post-hoc Dunnett's test, which compares all data with the control group. To compare two different groups, a student t-test was used. $P < 0.05$ was regarded as statistically significant.

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