Transdermal delivery of AT$_1$ receptor antagonists reduce blood pressure and reveals a vasodilatory effect in kidney blood vessels.

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Running title: Anti-hypertension patch

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Abstract

Background: The Renin Angiotensin System (RAS) is pharmacologically targeted to reduce blood pressure, and patient compliance to oral medications is a clinical issue. The mechanisms of action of angiotensin receptor blockers (ARBs) in reducing blood pressure are not well understood, and is purported to be via a reduction of angiotensin II signaling.

Objective: We aimed to develop a transdermal delivery method for ARBs (losartan potassium and valsartan) and to determine if ARBs reveal a vasodilatory effect of the novel RAS peptide, alamandine. In addition we determined the anti-hypertensive effects of the transdermal delivery patch.

Methods: *In vitro* and *in vivo* experiments were performed to develop an appropriate therapeutic system, promising an alternative and more effective therapy in the treatment of hypertension. A variety of penetration enhancers were selected such as isopropyl myristate, propylene glycol, transcutol and dimenthyl sulfoxide to obtain a constant release of drugs through human skin. Small resistance vessels (kidney interlobar arteries) were mounted in organ baths and incubated with an ARB. Vasodilatory curves to alamandine were constructed

Results: The *in vivo* studies demonstrates that systemic absorption of valsartan and losartan potassium using the appropriate formulations provides a steady state release and anti-hypertensive effect even after 24 hours of transdermal administration. No apparent skin irritations (erythema, edema) were observed with the tested formulations. We also show that blocking the AT_1 receptor of rabbit interlobar arteries *in vitro* reveals a vasodilatory effect of alamandine.

Conclusion: This study reveals potential mechanism of AT_1 receptor blockade via alamandine, and is an important contribution in developing a favorable, convenient and painless antihypertensive therapy of prolonged duration through transdermal delivery of AT_1 blockers.

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Graphical Abstract
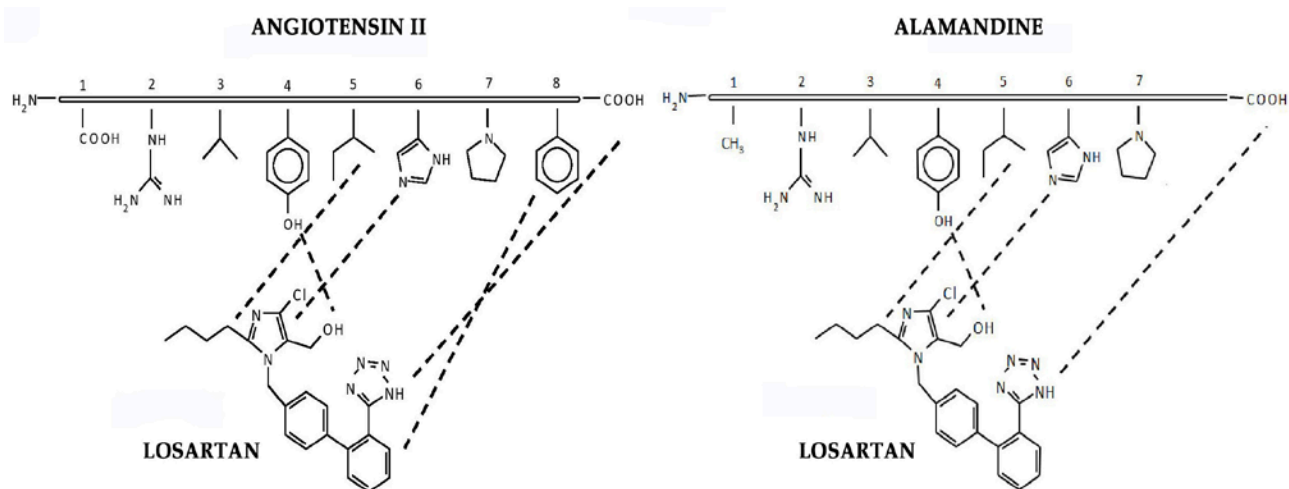
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INTRODUCTION

Transdermal drug delivery system (TDDS) is a novel approach for administration of drugs. They offer several important pharmacological advantages over conventional dosage forms, such as avoidance of first-pass metabolism by the liver, minimizing pain, controlled release of drug and prolonged duration [1, 2]. Currently, approximately 74 % of drugs are administered orally and have reduced efficacy due to first pass metabolism, as well as related side effects. As well, drugs for several diseases such as diabetes (insulin), multiple sclerosis (interferons) and cancer (taxol) taken by injections must be administered in a painless and effective manner [3]. In this regard, transdermal delivery of drugs can bypass these issues. For example, new transdermal drugs are used for multiple sclerosis (MBP_{85-99}, MOG_{35-55} and $PLP_{139-155}$) [4]. Thus, TDDS (transmucosal delivery systems) and controlled release systems have emerged to overcome disadvantages of the oral or injection route [5]. However, the stratum corneum (SC) is a barrier to the absorbtion of such drugs [6]. The characteristic requirements for a drug to be effective is a small molecular size and weight less than 500 Da, significant lipophilicity, efficacy in low plasma concentration and high degree of stability [7-9]. To enhance transdermal absorption of the active ingredient, drug derivatives, prodrugs, drug saturated systems and physical and chemical enhancers that facilitate permeation of the drug through the stratum corneum can be used [10, 11].

Herein, we used ethanol (EtOH), isopropyl myristate (IPM), propylene glycol (PG), transcutol (TCL) and DMSO as CPEs [11-13]. IPM is an ester, well-known to be an effective CPE for a wide variety of drugs including indapamide, S-almodipine, isosorbide dinitrate, lincomycin hydrochloride and terbutaline sulfate [8]. PG is a common excipient which promotes the flux of heparin sodium, verapamil hydrochloride and ketoprofen [11]. The combination of

TCL and PG increases clonazepam permeation [14]. DMSO is one of the earliest and most widely studied penetration enhancers. It is used to treat severe herpes infections of the skin and as a co-solvent in idoxuridine [11, 13]. In addition, nitroglycerine has been used as a patch for angina. A sartan as a patch will be beneficial to all RAS based hypertensive patients [15, 16]. The merits of a sartan patch is the continuous control of blood pressure, considering that in the morning patients appear to have increased hypertension [17]. Blood pressure could be monitored with suitable enhancers allowing appropriate sartan penetration and longer duration. In the current study we aimed to develop a transdermal delivery method for angiotensin receptor blockers (ARBs, ie. losartan potassium and valsartan), their anti-hypertensive properties and to determine whether ARBs reveal a vasodilatory effect of the novel RAS peptide, alamandine.

Valsartan and losartan potassium were chosen in this study. Valsartan is an orally active angiotensin II (ANGII) type 1 receptor blocker (ARB) and is prescribed for the management of hypertension. It is a lipophilic drug with low molecular weight (435.519 g/mol), available as a white, microcrystalline powder with a melting range of 105-110 °C with low oral bioavailability (25 %). The partition coefficient P is 0.033 (log P=1.499), suggesting that the compound is rather hydrophilic at physiological pH and half life is approximately 6 hours [18]. Losartan potassium, the first selective AT_1 blocker, has a low molecular weight (461.01 g/mol), available as white crystalline powder with melting point 183-184 °C with low oral bioavailability (33 %). The log P is 3.85 [19] and half life is approximately 2.1±0.70 hours [7].

Our interest in the effects of small RAS peptides and ANG II receptor antagonists [20-24] could be extended to congestive heart failure, as well as for the pathogenesis of diabetes and kidney disease [25, 26]. Selective blockade of the AT_1 receptor may prevent the known pathologic effects of ANG II associated with its stimulation, whilst allowing the positive effects

induced by the AT_2 receptor [21, 27, 28]. Previously we designed, synthesized and studied biological and physicochemical properties of peptides and non peptide mimetics of ANGII [20, 22, 23, 29, 30]. Furthermore, the reduction of blood pressure observed by ARBs is claimed to be due to the reduction in ANGII/AT_1R signaling, yet circulating ANGII is increased nearly 5-fold [31] and the kidney plays a significant role in hypertension in rat [32]. We propose that blockade of the AT_1 receptor could reveal other vasodilative effects in the vasculature, by enhancing the vasodilatory peptides of RAS, such as alamandine [33, 34] in kidney blood vessels. Alamandine, a novel heptapeptide of the RAS, has been demonstrated to enhance acetylcholine (ACh)-mediated vasodilation in New Zealand white rabbits *in vitro* [35] and antihypertensive effects in spontaneously hypertensive rats after oral administration [36].

MATERIALS AND METHODS

Materials

Valsartan and losartan potassium were a gift from pharmaceutical company, Vianex SA. PG, TCL, IPM, EtOH and phosphate buffer were purchased from Sigma Aldrich (Germany, purity > 98 %). HPLC grade water was obtained from distilled water passed through a milli-Q water purification system (Millipore Ltd., Bedford, MA, USA). HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were purchased from Panreac Química S.A. DMSO and trifluoroacetic acid (TFA) were of HPLC analytical grade and were purchased from Scharlau and Alfa Aesar respectively.

All chemicals for KREBS (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$ $7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.7 mM glucose), ACh (purity > 99 %), phenylephrine and candesartan (purity > 98 %) were purchased from Sigma Aldrich (USA). Alamandine (purity 99

%) was purchased from the Mimotopes (Notting Hill, Australia).

Animals

Wistar rats (aged 5 months) were purchased from Greek Institute of Pasteur and were bred in the animal experimental center of medical department of University of Patras. Animals were individually housed and maintained on a 12:12 light:dark cycle (lights on at 7am) at 25 ± 2 ^{o}C with *ad libitum* access to food and water throughout the experiment. All experimental protocols were conducted in accordance with the law of the European convention for the protection of animals used for experimental or other research purposes (2010/63/EU) and were approved by the institutional animal care and use committee at the school of Medicine, University of Patras, Greece (code EL13 BIO 04). Male New Zealand white rabbits (aged 3 months) were housed in individual cages and maintained at a constant temperature of 21 ^{o}C, where food and water were supplied *ad libitum*. These animals were approved by the Victoria University animal ethics committee (VUAEC#03/11) under the guidelines of the National Health and Medical Resaerch Council (NHMRC) Australia.

Preparation of human skin

Human live skin was collected from unused portion of human female patients from General University Hospital of Patras with consent. Subcutaneous adhering fat and connective tissues were carefully removed and the excised skin was cut to an appropriate size. Full thickness human skins were stored at -20 ^{o}C until the day of the experiment.

Preparation of the formulations

Preparation of valsartan formulations V2, V3 and V5 (Table 1): Valsartan (0.625 g) was dissolved in EtOH (0.75 g) and the mixture was vortexted for 30 seconds. DMSO (0.125 g) was added and the mixture vortexed to achieve a homogenous solution. Additionally, accurately weighed quantity of other ingredients: PG (0.75 g), IPM (0.25 g); IPM (0.25 g), TCL (0.75 g); PG (0.25 g), IPM (0.75 g) were added into formulations V2, V3 and V5 respectively. A 2-phase formulation was produced after the addition of IPM.

Preparation of valsartan formulations V1 and V4 (Table 1): Valsartan (0.625 g) was dissolved in EtOH (0.625 g) and the mixture was mixed for 30 seconds using a vortex mixer. IPM (1.25 g) and PG (1.25 g) were added into V1 and V4 formulations respectively to achieve the final formulations. A 2 phase formulation was produced after the addition of IPM.

Preparation of losartan formulations L1-L5 (Table 1): Losartan potassium (0.625 g) was dissolved in H_2O (0.498 g) and the mixture was mixed for 30 seconds using a vortex mixer after which EtOH (0.287 g) was added and the mixture mixed to achieve a homogenous solution. Additionally accurately weighed quantity of other ingredients: PG (0.125 g), IPM (0.839 g), TCL (0.125 g); PG (0.125 g), IPM (0.964 g); IPM (1.089 g); PG (1.089 g); IPM (0.544 g), PG (0.544 g) were added into formulations L1 - L5 respectively. A 2-phase formulation was produced following the addition of IPM.

Losartan was used in these studies based on a feasibility report that we conducted that suggested that the transdermal delivery of losartan was feasible, and thus it was used over telmisartan which is more lipophlic than losartan or candesartan. Estimations were based on physicochemical properties of losargan, its overal dose and bioavailability. Moreover, valsartan was selected since it had similar molecular weight with losartan, was more potent, with lower daily dose, and both sartans were available as generics for further development.

Alamandine is a heptapeptide formed from the removal of the 8th peptide from angiotensin A, possibly by ACE2. Alamandine differs from angiotensin (1-7) in the primary amino acid, alanine (which contains a methyl group on the amino end), compared to aspartate, (which contains a carboxy group), respectively [37]. Extensive simulation and modeling studies between the octapeptide ANGII and Losartan, based on SAR and NMR findings, had previously shown mimicry of the ANGII critical amino acid side chains (Tyr, Val, His, Phe) and the C-terminal carboxylate with Losartan's pharmacophoric groups (-CH$_2$OH, imidazole ring, butyl side chain, tetrazole) (Scheme 1a) [38]. Deletion of the Phe residue as in Alamandine, deprives the heptapeptide from its agonist property rendering the vasodilatory effects (Scheme 1b). These studies show the importance of the Phe aromatic ring at position 8 for agonist hypertensive activity.

Instrumentation – Liquid Chromatography

All analyses were performed on a Waters Alliance HPLC^{TM} system (Waters 2695 separation module equipped with waters 2996 photodiode array detector and data acquisition empower 2). Chromatographic separation was performed on a XBridge^{TM} C18 (4.6mm ID*150 mm length) column with 3.5 μm particle size [39]. Under the described experimental conditions, the peak was well defined and free from tailing.

Preparation of standard solutions and quality control samples

Freshly prepared stock solutions of valsartan and losartan (1 mg/ml) were prepared by dissolving 10 mg of the compound in 10 ml of MeOH and purified H$_2$O, respectively. Nine working standard solutions of the analyte at concentrations of 0.1, 0.2, 1, 2, 10, 20, 50, 100 and

200 µg/ml were prepared by further dilution of the stock solution with appropriate volumes of H$_2$O. Low, medium and high quality control (QC) samples of the analytes at concentrations of 8 µg/ml, 90 µg/ml and 160 µg/ml were similar and prepared by further dilution of the stock solution with appropriate volumes of H$_2$O for the evaluation of the methods.

Preparation of internal standard and calibration model

The concentration range of the 2 compounds for the construction of the calibration curves was between 0.1 and 200 µg/ml, using the internal standard (IS) method. Losartan potassium (20 µg/ml) and valsartan (40 µg/ml) were used as IS for valsartan and losartan, respectively.

The construction of the calibration curve was based on the analysis of the calibration standards (n=4) at the 9 concentration levels ranging from 0.1 to 200 µg/ml and plotting the peak area ratios of the analyte to IS against the nominal calibration standard concentration. Three different calibration curves (n=3) were prepared during two weeks (Table 2). Linearity was evaluated by linear regression analysis, which was calculated by the least-square regression analysis. LOD was determined as the sample concentration obtaining a peak area of 3 times the noise level and LOQ as the sample concentration obtaining a peak area of 10 times the noise level [40] and the lower point of the calibration curve is used for estimating [41].

Intra- and inter- day precision and accuracy

Precision of the analytical method was defined as relative standard deviation (% RSD) and was evaluated in terms of intra-day and inter-day precision for four samples (n=4) at concentrations of QC samples at three levels (low QC, medium QC and high QC). Accuracy of the method was determined by relative error (% RE) [42].

In vitro skin permeability studies

A vertical Franz-type glass, jacketed diffusion cell system with a magnetic stirrer and a thermostatic water bath was used for the studies [8, 43, 44]. The full-thickness excised human skin (bround to room temperature) was mounted on the top of the Franz diffusion cell (vertical available diffusion area, 1.77 cm^2; volume of receiver cell, 12 ml) and was fastened with a rigid clamp. The temperature was maintained at 32 ± 1 ^{o}C using a thermostatic water bath. The SC side of the skin sample was facing upward into the donor compartment and the dermal side was facing downward into the receptor compartment. The receptor compartment was filled with phosphate buffered saline (pH 7.4) and stirred by magnetic bar to ensure adequate mixing and maintenance of sink conditions. For each experiment, the donor compartment was loaded with 300 µl of the formulation of the drug to the SC side of the skin. Samples (480 µl) were withdrawn every 3, 6, 8, 24, 30 hours and equal volume of blank solution was immediately added. Six replicates of each experiment were performed. At the end of the experiment the skin remained intact and undamaged. The standard curve was used for estimating the concentration of valsartan and losartan in samples using HPLC.

Mechanistic assessment of AT$_1$ receptor blockade in organ bath studies

Rabbits were used to assess the mechanism of AT$_1$ receptor blockade due to their sensitivity to ANGII blood vessel constriction similar to human arteries [45, 46]. Rabbits were killed by exsanguination after anesthesia with 4 % isoflurane, followed by the extraction of interlobar arteries from kidneys (VUAEC#03/11). Interlobar arteries were cleaned of fat and connective tissue then cut into 3 mm arterial rings and mounted onto organ baths attached to

force displacement transducers in order to measure the response of arterial rings in grams (OB8, Zultek Engineering, Melbourne, Australia [47]). The organ baths were filled with KREBS which was at a constant temperature of 37 ºC with continuous bubbling of carbogen (95 % O_2 and 5 % CO_2). Phenylephrine (Phen) was used to pre-constrict the arterial rings until a plateau was reached. Candesartan (10^{-5} M) [48, 49] was added for 5 mins prior to the first dose of alamandine dose response curve. ACh was added after the final dose of alamandine to indicate functional arterial rings. Candesartan was used in organ baths as it has direct effects on renal blood vessels [48, 49] as well as being commonly used in animal models of blood pressure studies [48].

In vivo biological evaluation

The time-dependent antihypertensive efficacy of valsartan and losartan potassium formulations via transdermal administration in Wistar rats (370 ± 80 g) was evaluated by the modulations of the hypertensive response elicited by subcutaneous bolus injections of ANG II (50 μg/kg body weight) were recorded in eight Wistar rats (370 ± 80 g) using at 0, 3, 6, 8 and 24 hours. The mean arterial blood pressure was recorded using a Coda, non-invasive blood presure system (Kent Scientific) [50]. Fourty eight hours prior to the treatment, hair from the dorso-lumbar region at two contralateral sites of each rat was depilated. A volume of 300 μl of V2 formulation for valsartan (n=6), L1 formulation for losartan (n=6) and placebo (n=2) were applied transdermally in a depilated area, of approximately 10 cm^2, and a membrane was over it to secure the formulation in place and prevent the evaporation of the volatile solvents. In addition, the treated site was covered with gauze and secured at the margins to prevent leakage of the test substance and to ensure that the rats did not bite and remove the formulation.

Data analysis

The permeation of valsartan and losartan using different enhancers was assayed for 30 hours and plots of the cumulative amount of the permeated drug ($\mu g/cm^2$) through human skin were plotted versus time (hours). The transdermal flux (J, $\mu g/cm^2*h$), the rate of change of the cumulative amount of drug passes per unit area and time through the skin, was calculated from the steady-state part of the curve. Lag time (T_{lag}) is obtained by the extrapolation of the linear portion to the x-axis [2]. The release data was fitted into various mathematical models using software to know which mathematical model will best fit to obtained release profiles. To determine the effects of penetration enhancers on the skin permeation of valsartan and losartan GraphPad Prism (Version 7, GraphPad Software Inc, USA) was used to perform two-way ANOVA (dose vs flux) followed by Tukeys multiple comparisons test to determine significance between each group, with $p < 0.05$ considered significant. For the organ bath procedures, the response given in grams was translated into % of response against each cumulative doses of alamandine. GraphPad Prism was also used to perform a two-way ANOVA (dose vs response) repeated measures followed by a Sidak's multiple comparisons post hoc test to determine significances between doses of alamandine. $p < 0.05$ was considered a significant.

RESULTS

Method Validation

The calibration standard samples analyzed by the developed analytical methodology showed that the retention time of valsartan and losartan was 9.43±0.03 min and 4.85±0.03 min, respectively. Over the concentration range from 0.1 to 200 μg/ml regression analysis indicated that there was an excellent linearity between UV absorption and valsartan and losartan concentrations (Table 2). The limit of quantification (LOQ) of the developed methodology was 0.1 μg/ml and the limit of detection (LOD) was 0.03 μg/ml. The methods were evaluated in terms of intra-day precision and accuracy by assaying (n=3) QC samples at low QC (8 μg/ml), medium QC (90 μg/ml) and high QC (160 μg/ml). Table 3 shows the precision and accuracy of the analytical method of valsartan and losartan calculated in values of % RSD and % RE.

Effects of penetration enhancers on the skin permeation of valsartan and losartan

The effects of CPEs on the permeation of valsartan and losartan from formulations (V1-V5, L1-L5) through full thickness human skin are summarized in Table 4. It is well known that the penetration of a drug through rat skin is considerably greater than that through human skin [8]. Therefore, human skin was used to obtain a better estimation of the drug permeation in humans. Among all portions, V2 and V1 for valsartan (Fig. 1) with a flux value of J=0.5024 μg/cm^2*h and J=0.3774 μg/cm^2*h, respectively (Table 4) and L1 for losartan (Fig. 2) with a flux value of J=0.2652 μg/cm^2*h (Table 4) had the most conspicuous effect. Accordingly, formulation V2 which contained 30 % w/w PG, 10 % w/w IPM and 5 % w/w DMSO showed a significant increase of permeation flux within 3 hours ($p < 0.0001$ V2 compared to V1, $p < 0.01$ V2 compared to V3 and $p < 0.01$ V2 compared to V5) which continued to significantly increase

up to 30 hours ($p < 0.05$ V2 compared to V1, $p < 0.0001$ V2 compared to V3, $p < 0.0001$ V2 compared to V4, and $p < 0.0001$ V2 compared to V5) (Fig. 1). In addition, L1 which contained 33.57 % w/w IPM, 5 % w/w PG and 5 % w/w TCL showed significant increase of permeation flux compared to L2, L3, L4 and L5 within 6 hours and remained significant up to 30 hours ($p < 0.0001$) (Fig. 2). It is reported that the purposed formulation containing two or three enhancers showed significantly enhanced permeation. Specifically, the addition of PG, IPM and DMSO (V2) and the addition of PG, IPM and TCL (L1) resulted in a synergistic penetration enhancement effect on valsartan and losartan, respectively. In general, enhancers may change the structure of the lipids in the SC, denature the skin proteins and changes the properties of the horny layer, and as a consequence the SC becomes more permeable for drugs [51-53]. The enhancement resulting from the presence of IPM is connected with its increased fluidity in the lipids of the skin as well as with the lipid disruption due to high affinity between IPM and the components of the skin [8, 51].

In vivo **evaluation studies**

In order to investigate the *in vivo* transdermal process of valsartan and losartan, the more efficient formulation resulting from the *in vitro* studies was estimated by the effects of antihypertensive evaluation in Wistar rats (Fig 3). Mean arterial blood pressure (MABP) was recorded after 3, 6, 8 and 24 hours of subcutaneous bolus injections of ANGII in order to evaluate the antihypertensive activity of valsartan and losartan following transdermal delivery to the systematic circulation. As shown in Fig. 3, losartan showed a significant decrease in MABP by 24 hours ($p < 0.00001$), however, valsartan was more potent showing significant reduction in MABP within 3 hours ($p < 0.05$) which remained significant at 6, 8 and 24 hours ($p < 0.01$)

following administration. Since there was no observable skin irritation in the animals during the study, these 2 transdermal formulations can be used for further development.

Assessment of AT_1 receptor blockade in organ bath studies

In order to determine the pathway of which blocking the AT_1 receptor exerts vasodilatory effects, we conducted a dose response curve to alamandine with candesartan (an active *in vitro* ARB). The addition of candesartan had no effect on vasoconstriction caused by phenylephrine. Alamandine induced direct vasoconstriction on interlobar artery in a dose dependent manner (10^{-8} M, 10^{-7} M and 10^{-6} M corresponded to 5.4 ± 1.3 %, 12.2 ± 1.9 % and 15.2 ± 0.9 % of constriction, respectively). However, candesartan revealed a vasodilatory effect of alamandine in the interlobar arteries, to 1 ± 1.7 %, -6.7 ± 3.0 % ($p<0.001$) and -10.1 ± 5.0 % ($p<0.001$) (Fig. 4A). Real time trace of the experiments are also shown (Fig. 4B).

DISCUSSION

In this study, we show that ARBs can be formulated so that they are better absorbed through the skin to reduce blood pressure, possibly via a vasodilatory effect of alamandine in kidney blood vessels.

In vitro **evaluation of Valsartan**

In this study, a combination of penetration enhancers were used to evaluate the drug release of valsartan and losartan potassium. Valsartan is a poorly water - soluble drug with poor oral bioavailability (approximately 25%). Permeation enhancers such as IPM, EtOH, DMSO improves drug solubility and penetration into the skin. The formulations V1 and V4 constituted

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of EtOH as main solvent and IPM and PG respectively. The V2 formulation, which contained a mixture of these two enhancers showed a significant increase of permeation flux. From the data obtained from V3 and V5 formulation, changing the proportions of enhancers (PG and IPM), showed less permeation flux indicating the necessity of PG. However, the formulation V2 showed to be the most effective delivery. The presence of DMSO, PG and higher percentage of EtOH compared to V1 formulation, improved drug solubility and changed the thermodynamic activity of valsartan. The solubility of valsartan in stratum corneum was improved and this caused an overall improvement in permeation behavior of valsartan across the human skin. It is likely that these conventional chemical enhancers disrupt the highly ordered bilayer structures of the intracellular lipids found in stratum corneum by inserting amphiphilic molecules into these bilayers to disorganize molecular packing.

In vitro evaluation of Losartan potassium

There are several publications in the literature demonstrating that molecules in neutral form have better permeation rates compared to charged molecules in transdermal transport [54]. Human skin is hydrophobic and the salt from the original drug molecule has poor permeability across the skin as compared to its neutral or base form. However in this study we have chosen losartan potassium, as the best candidate to be tested. Losartan potassium yielded additional advances compared to neutral form. In particular the salt form is soluble in water which was used as a solvent in the formulations tested. In addition, losartan potassium is the active pharmaceutical ingredient that has been used in numerous transdermal patches designed and demonstrated in the literature [55-57].

Losartan potassium is freely soluble in water with oral bioavailability of 33 % due to first pass metabolism in liver. It is also soluble in alcohols. All produced formulations of losartan potassium contained water and EtOH as main solvents in equal proportions. EtOH was also used to enhance penetration. DMSO was avoided to be used in high concentrations in the prepared formulations due to the known toxic side effects. The potential advantage was that the produced formulations did not have any pathological effects on the skin. The formulations L3 and L4 (solvents and IPM and PG respectively), were the guide to modify and produce improved formulation consisted of a mixture of IPM and PG. According to the analysis the formulation L1 showed the best permeation, which appears to be twice as high compared to formulations L2 and L5. The presence of TCL in formulation L1, -not in the other formulations- indicates the importance of this ingredient for enhancing permeability and showing a constant release profile. It seems that the lipophilic alkyl chain length of TCL is an important parameter in the promotion of penetration enhancement. It is reported that the lipid layers of stratum corneum play a significant role in skin permeation for lipophilic drugs.

***In vivo* studies for valsartan and losartan potassium**

The antihypertensive effect of transdermal administration of valsartan and losartan potassium were evaluated in Wistar rats. Animals treated with formulations V2 (valsartan) and L1 (losartan), showed a significant decreased in MABP even after 24 hours of administration. Transdermal delivery is a "slow intravenous infusion" with 100 % bioavailability. Findings from the *in vivo* studies indicated that both sartans had the ability to penetrate through rat skin. A therapeutic response was achieved 3 hours after administration. Furthermore, no skin irritation was observed in both cases which indicated that these particular formulations could be used for

further development. V2 significantly lowered blood pressure as early as 3 hours which remained until 24 hours and L1 was shown to lower blood pressure induced by ANGII as early as 3 hours although not significant, which was further decreased and showed significance by 24 hours.

In vitro studies for the mechanism of AT$_1$ receptor blockade

As blocking the AT$_1$ receptor results in beneficial antihypertensive effects, we used rabbit interlobar arteries to determine if other vasodilators could be involved in this hypotensive effect. It is well established that the endothelium layer of arteries plays a major role in exerting beneficial effects such as vasodilation via releasing nitric oxide [58], suggesting that candesartan, losartan and valsartan as AT$_1$ receptor blockers, could cause the release of nitric oxide in order to induce vasodilation to exhibit antihypertensive effects. However, in our present study we have shown that upon AT$_1$ receptor blockade, the vasodilative effect of the RAS heptapeptide, alamandine, is revealed within renal interlobar arteries. However, in animals that respond poorly to angII, such as rat aorta, alamandine is not a vasoconstrictor but instead a vasodilator [36], supporting our results [59, 60]. Interestingly, in rabbit aorta, we have shown that alamandine does not constrict nor dilate rabbit aorta (unpublished observation), but does enhance ACh mediated vasodilation [35], demonstrating that each vascular bed could respond differently to peptides.

The vasodilatory beneficial antihypertensive effect of alamandine is due to its structural features, which are in line with our previous studies about the role and importance of phenylalanine aromatic amino acid at position 8 triggering agonist activity [26, 27, 29, 61, 62]. Structure activity studies on Angiotensin analogues have shown that replacement of the Phe aromatic residue at position 8 with an aliphatic amino acid such as Ile, results in an antagonist

peptide with antihypertensive activity. Deletion of the Phe residue at position 8 of the octapeptide ANG II results in the novel RAS heptapeptide Alamandine, as well as Aspartic Acid at position 1 replaced by Alanine. Both modifications, replacements of Phe at position 8 with an aliphatic amino acid or deletion of the Phe, amino acid result in peptides with antagonists antihypertensive vasodilatory effects. Biological effects are in line with the documented evidence that the Phe amino acid in position 8 is critical for agonist hypertensive activity. Our laboratory has been engaged for many years in the conformation analysis study of ANGII. Its superagonist $[Sar^1]$ ANGII in DMSO supports a bioactive conformation characterized by (a) a Tyr^4-Ile^5-His^6 bend, (b) a major His^6-Pro^7 *trans* conformer, (c) a cluster of the side chain aromatic rings of the triad key amino acids Tyr^4, His^6, Phe^8, which drives the formation of the charge relay system between Tyr^4 Hydroxyl, His^6 imidazole and Phe^8 carboxylate, analogues to that found in serine proteases. This relay system appears to be responsible for ANGII and $[Sar^1]$ ANGII biological activity. Disruption of this system, which occurs upon the replacement of the Phe ring with an aliphatic or deletion of the Phe amino acid in Angiotensin, results in antagonists antihypertensive beneficial effect as in the case of alamandine. Alamandine is a novel peptide of the RAS system and evidence is accumulating to show a vasodilatory beneficial effect.

CONCLUSION

In summary, we demonstrated the effect of various formulations of the transdermal delivery of valsartan and losartan potassium. The formulations V2 and L1 for valsartan and losartan potassium respectively have great potential of transdermal administration, promising an alternative and more effective therapy in the treatment of hypertension. Moreover the results indicate a controlled delivery rate through the skin suggesting potential for conversion to a

matrix system. Results also indicate that another possible mechanism by which AT_1 blockade exerts its vasodilatory/hypotensive effects is through the vasodilatory effect of alamandine in the kidney.

In conclusion, transdermal administration of sartans could be a feasible alternative route for prolonged hypertensive therapy, acting via ANGII type 1 receptor blockade and alamandine vasodilation.

List of Abbreviations

ACh, acetylcholine; ANGII, angiotensin II; AT_1, angiotensin II type 1 receptor; AT_2, angiotensin II type 2 receptor; CPE, chemical penetration enhancers; DMSO, dimethyl sulfoxide; EtOH, ethanol; h, hour; HPLC, high performance liquid chromatography; IPM, isopropyl myristate; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; MABP, mean arterial blood pressure; MeCN, acetonitrile; MeOH, methanol; min, minutes; PG, propylene glycol; Phen, phenylephrine; RAS, renin-angiotensin system; RE, relative error; RSD, relative standard deviation; SC, stratum cornuem; SD, standard deviation; SEM, standard error of the mean; TDDS, transdermal drug delivery systems; TCL, transcutol; TFA, trifluoracetic acid; QC, quality control.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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FIGURE LEGENDS

Scheme (1). Schematic representation of (A) angiotensin II (ANGII) and losartan, and, (B) alamandine and losartan.

Fig. (1). *In vitro* diffusion of valsartan versus time in hours, using franz diffusion cells and full-thickness human skin. Each point and data represents the mean ± standard error of the mean (SEM) of 6 replicates. * = $p < 0.05$,** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = no significance. Statistics shown in each are for V2 compared to V1, V3, V4, V5.

Fig. (2). *In vitro* diffusion of losartan versus time in hours, using franz diffusion cells and full-thickness human skin. Each point and data represents the mean ± standard error of the mean (SEM) of 6 replicates. * = $p < 0.05$,** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = no significance. Statistics shown in each are for L1 compared to L2, L3, L4, L5.

Fig. (3). Mean arterial blood pressure (MABP) of Wistar rats (n=6/group), showing normal MABP, the increase of MABP after ANG II administration (ANGII) and the MABP after 3, 6, 8 and 24 h of sartan transdermal administration. Each point and data represents the mean ± standard deviation (SD) of n=6 rats. * = $p < 0.05$,** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = no significance (upper statistics losartan compared to ANGII control, lower statistics valsartan compared to ANGII control).

Fig. (4). (A) Blocking the AT_1 R in *in vitro* reveals the vasodilative effect of alamandine in rabbit resistant arteries (interlobar). (B) Real time traces of alamandine dose response curve (in

green) and in candesartan incubated interlobar rings (red) after phenylephrine constrictions. ACh demonstrates that the endothelium was intact and therefore the blood vessels were functional. Each point and data represents the mean ± standard error of the mean (SEM) of n=3 rabbits. $* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001$, ns = no significance
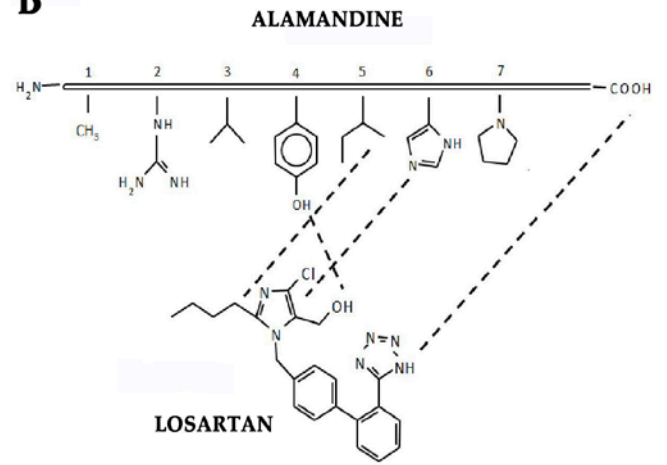
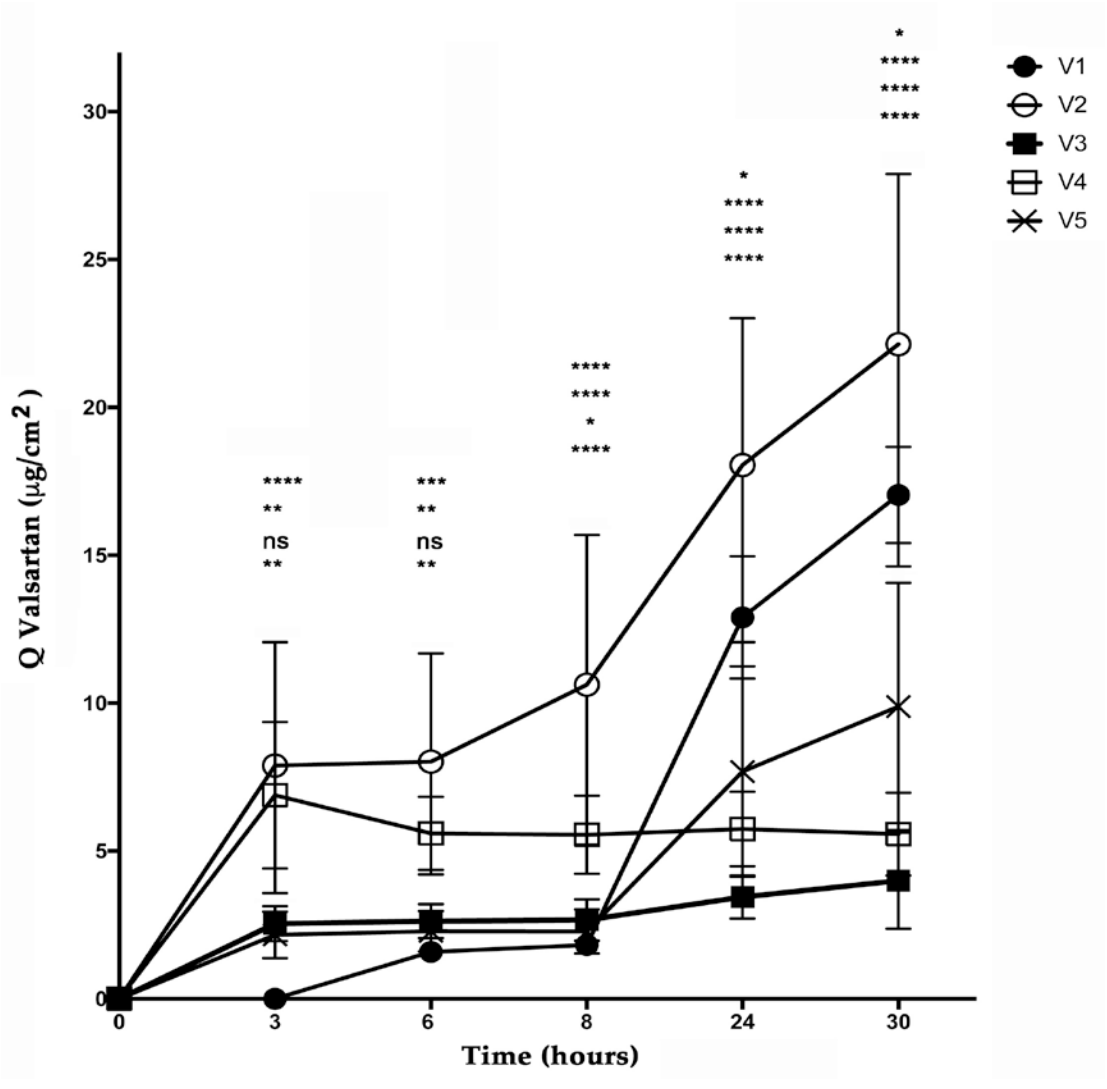**Scheme (1).**
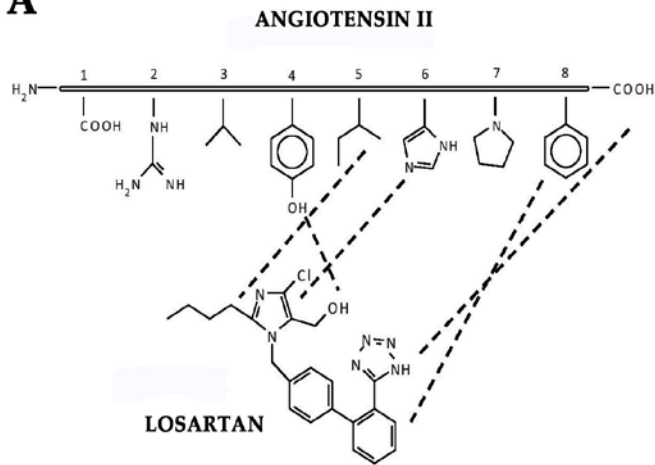
Fig. (1).

Fig. (2).

Fig. (3).

Fig. (4).

A

B
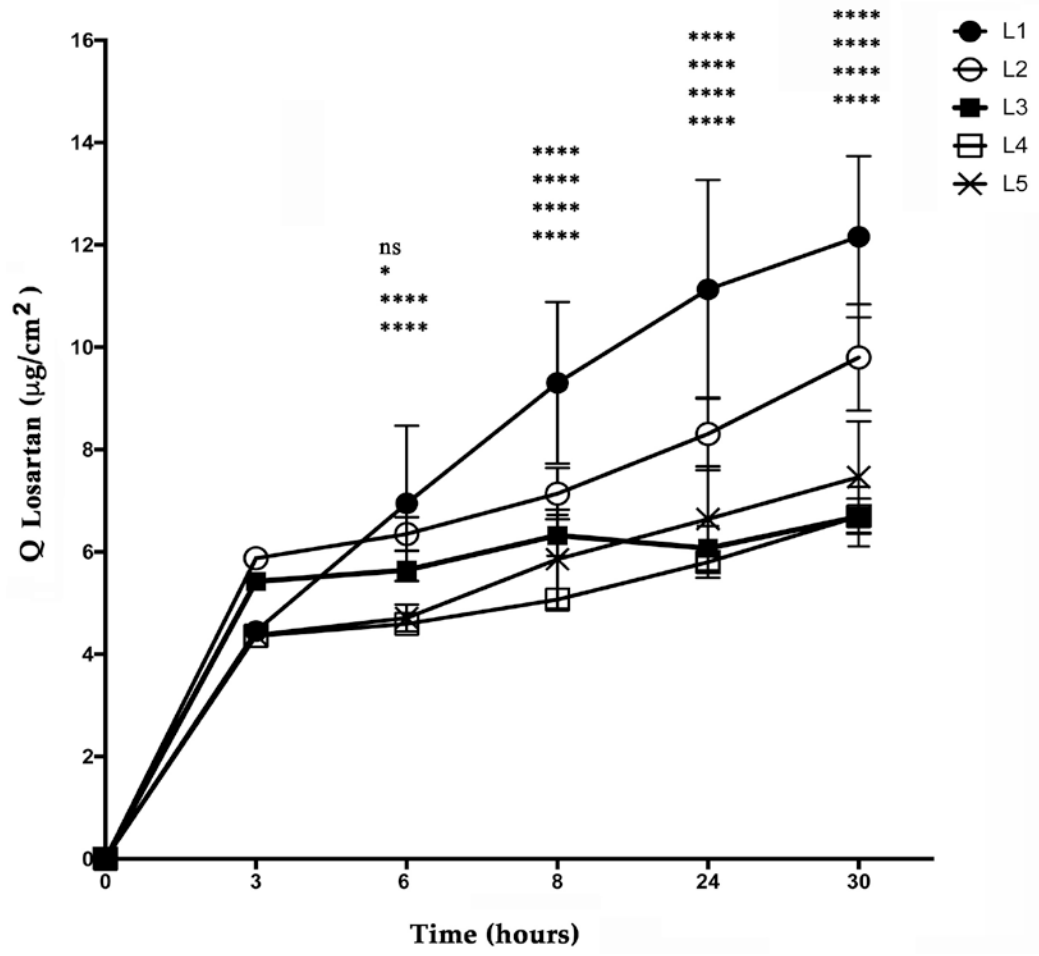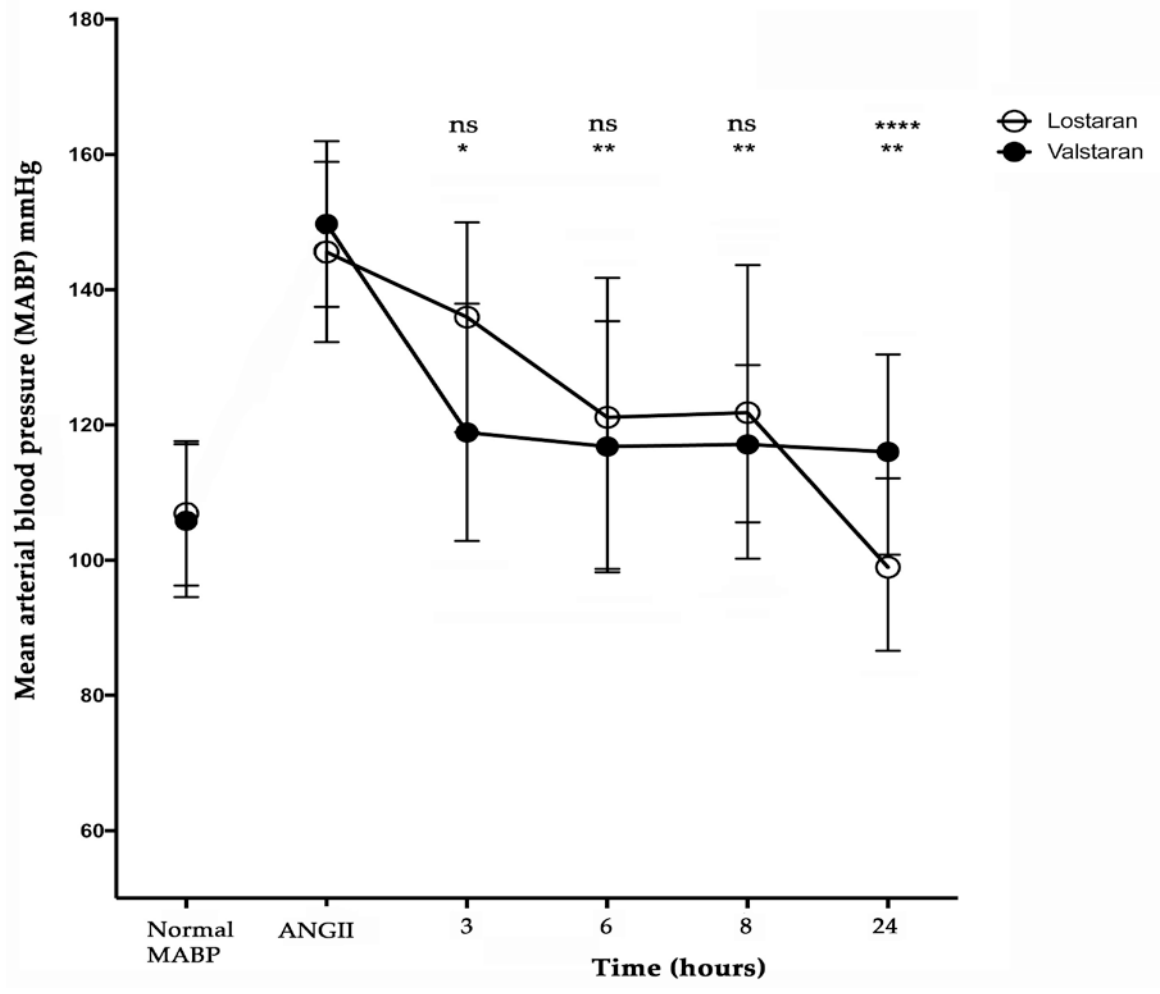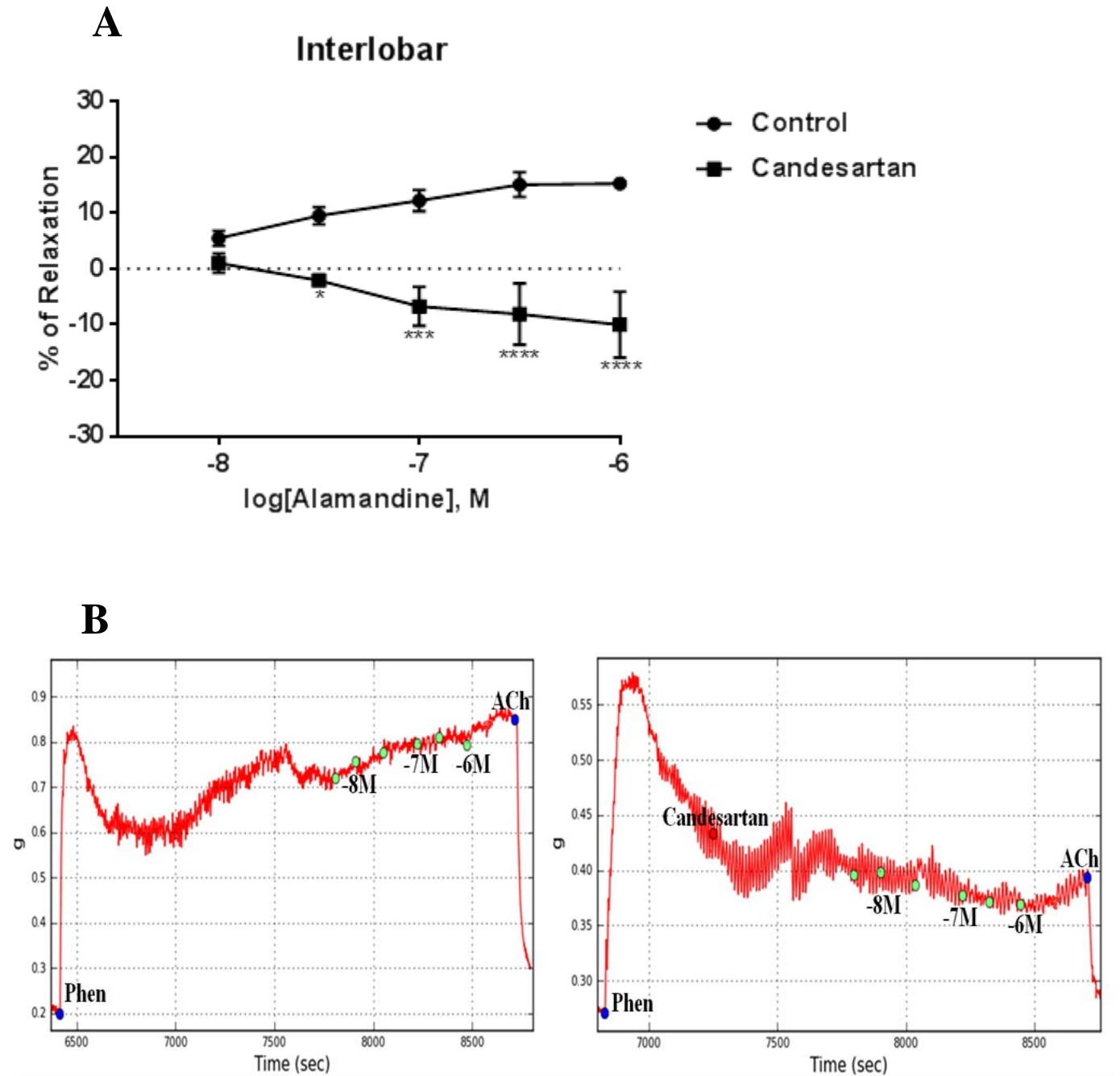
Table 1
Composition of valsartan and losartan formulations

A	Formulations									
	% w/w					g				
Ingredients	V1	V2	V3	V4	V5	V1	V2	V3	V4	V5
Valsartan	25	25	25	25	25	0.625	0.625	0.625	0.625	0.625
EtOH	25	30	30	25	30	0.625	0.750	0.750	0.625	0.750
PG	-	30	-	50	10	-	0.750	-	1.250	0.250
TCL	-	-	30	-	-	-	-	0.750	-	-
IPM	50	10	10	-	30	1.250	0.250	0.250	-	0.750
DMSO	-	5	5	-	5	-	0.125	0.125	-	0.125
Batch Size	100	100	100	100	100	2.5	2.5	2.5	2.5	2.5

B	Formulations									
	% w/w					g				
Ingredients	L1	L2	L3	L4	L5	L1	L2	L3	L4	L5
Losartan	25	25	25	25	25	0.6250	0.6250	0.6250	0.6250	0.6250
EtOH	11.5	11.5	11.5	11.5	11.5	0.2875	0.2875	0.2875	0.2875	0.2875
PG	5	5	-	43.57	21.78	0.1250	0.1250	-	1.0893	0.5445
TCL	5	-	-	-	-	0.1250	-	-	-	-
IPM	33.57	38.57	43.57	-	21.78	0.8393	0.9643	1.0893	-	0.5445
H$_2$O	19.93	19.93	19.93	19.93	19.93	0.4983	0.4983	0.4983	0.4983	0.4983
Batch Size	100	100	100	100	100	2.5	2.5	2.5	2.5	2.5

Table 2
Inter-day slopes, intercepts and square correlation coefficients (r^2) of calibration curves (n=3)

	Slope		Intercept		r^2	
	Valsartan	Losartan	Valsartan	Losartan	Valsartan	Losartan
1	0.9376	1.1313	0.0267	0.0114	0.9984	0.9997
2	1.0143	1.1022	0.0079	0.0045	0.9995	0.9994
3	0.9723	1.0534	0.0071	0.0220	0.9999	0.9994

Table 3

Precision (% RSD) and accuracy (% RE) data for valsartan and losartan

Actual concentration	Detected concentration (mean ± SD, n=4)	% RSD	% RE
Valsartan	Intra-day		
8 μg/ml	7.59±0.13	1.68	-5.14
90 μg/ml	87.74±1.13	1.28	-2.51
160 μg/ml	159.29±2.31	1.45	-0.44
	Inter-day		
8 μg/ml	8.07±0.82	10.16	0.92
90 μg/ml	86.93±1.31	1.50	-3.41
160 μg/ml	166.60±10.43	6.26	4.13
Losartan	Intra-day		
8 μg/ml	8.37±0.12	1.41	4.64
90 μg/ml	84.74±0.65	0.77	-5.84
160 μg/ml	153.97±1.82	1.18	-3.77
	Inter-day		
8 μg/ml	7.94±0.37	4.67	-0.71
90 μg/ml	83.67±1.91	2.28	-7.03
160 μg/ml	156.97±5.00	3.18	-1.89

Table 4

Effects of penetration enhancers on the permeation of valsartan and losartan

Formulations	Flux [μg/(cm^2*h)]	Lag Time (h)
V1	0.3774	1.0029
V2	0.5024	5.9960
V3	0.0445	2.3656
V4	0.0097	5.5077
V5	0.0241	2.1073
L1	0.2652	5.2518
L2	0.1080	5.8099
L3	0.0225	5.6345
L4	0.0656	4.2871
L5	0.0999	4.3721