Atenolol is a beta-adrenergic receptor blocking agent without membrane stabilizing or intrinsic sympathomimetic activity, which has been used for the treatment of hypertension. It is reported that, in case of oral administration, atenolol can induce side effects such as diarrhea, ischemic colitis and mesenteric arterial thrombosis. Therefore, development of transdermal drug delivery of atenolol maintaining proper blood level for a long time without adverse effects of frequent oral administration is very important (1).

Transdermal route shows certain biopharmaceutical benefits such as increasing bioavailability and therapeutic efficacy bypassing hepatic first-pass metabolism. This may be accompanied by the longer dosing frequency required for a chronic treatment and, thus, improved adherence (2). In recent years, owing to advantages offered by transdermal administration and the extensive use of beta blockers in the treatment of various cardiovascular disorders, some authors have studied percutaneous permeation and transdermal drug delivery of atenolol (1-5).

Even if transdermal route shows certain benefits, the barrier function of stratum corneum necessitates to enhance the transdermal delivery of therapeutic agents (6). One approach is to use chemical penetration enhancers, which can decrease the integrity of the skin barrier. An alternative strategy to facilitate transport is iontophoresis, which can enhance and/or control the delivery of molecules through the skin from appendageal pathways by applying a low-density electrical current (from 0.1 to 0.5 mA/cm²) (6, 7). Iontophoretic transport promotes penetration of small molecular weight cationic drugs into skin by both electrorepulsion and electroosmotic flow. Iontophoresis also promotes penetration of drugs through increased skin permeability (7-9). At physiological pH, the skin is negatively charged and cation perme-selective (7, 10). As the hydrophilic, atenolol has one positive charge at physiological pH resulting in a limited passive permeability and iontophoresis could be a suitable way to enhance transdermal delivery of this compound (11).
The use of polymethacrylate polymers as matrix polymers for transdermal systems has been reported by several authors (5, 12-15). The criteria of choice for these polymers were their high capacity for incorporating drugs and skin toleration (16). Drug containing films of water-insoluble polymers are generally prepared by casting and drying organic drug-polymer solutions or suspensions (17).

In this study, Eudragit E 100 and Eudragit NE 40D were used to prepare atenolol films (1.5 mg/cm²) by film casting method (1, 12, 13). Low-density direct currents of 0.1 and 0.5 mA/cm² were used to show the effect of iontophoresis on the in vitro release of atenolol from the films and the effect of 0.5 mA/cm² current density was compared with the effect of chemical enhancer oleic acid by in vivo studies.

**EXPERIMENTAL**

**Materials**

Atenolol was kindly donated from Abdi Ibrahim (İstanbul, Turkey) and sotalol was donated from Adeka (Samsun, Turkey). Silver electrodes (diameter 1.0 mm and purity 99.9%) and constant current source (Aselsan, Ankara, Turkey) were obtained from University of Ankara, Faculty of Medicine. Ketamine hydrochloride was kindly donated by Pfizer (Turkey). Eudragit E 100 and NE 40D polymers (ROEHM Pharma, Darmstadt, Germany), triacetine (Sigma), HEPES (Fluka), (-)-menthylchloroformate (Fluka), cellulose acetate membranes (Sartorius), oleic acid (Merck) and male, 250-300 g Wistar rats (Başkent University, Ankara, Turkey) were used. Milli-Q Water (Millipore, Bedford, MA, USA) was used in the preparation of the mobile phase for HPLC. All other chemicals were of analytical or HPLC grade.

**Preparation of the films**

Films composed of different ratios of Eudragit E 100 and NE 40D were prepared by film casting method. Each film was containing atenolol and triacetine, which were calculated from dry weight of the polymers per area as 5% w/w and 20% w/w, respectively. An accurately weighed amount of atenolol was dissolved in 2-propanol or methanol and was homogeneously dispersed in polymer-plasticizer mixture which was previously dissolved in acetone. The final mixture was stirred magnetically for 60 min at 500 rpm and was poured onto a glass dish covered with aluminium foil. The solvent was allowed to evaporate at room temperature overnight and then the matrices were stored at 25°C, 50% relative humidity conditions for 4 days (12, 13, 18, 19). The amounts of polymers and excipients used in the formulations are shown in Table 1.

**Intrabatch variation of the formulations**

In order to observe the effect of preparation on intrabatch variation, standart deviations of the thickness and atenolol contents of the films were evaluated. Thicknesses of the films were predicted as 0.300 mm and atenolol contents of the films were 1.5 mg/cm², theoretically. The study was done on four batches for each formulation and repeated three times for each batch.

**Determination of thickness of films**

Films were cut with a circular metallic die of 1.2 cm internal diameter to give an area of 1 cm² from different sides of the dish (n = 3) and thickness of these films was determined by micrometer (NSK, Japan), (Table 1).

**Determination of atenolol content in the films**

In order to show the uniformity of drug distribution in the dishes, discs were cut with a circular metallic die of 1.2 cm internal diameter (n=3) from each batch and were dissolved in 50 mL of alcohol for 1 h by magnetic stirring. The solution was analyzed using a spectrophotometer (Shimadzu 1202, Japan) at 274 nm and atenolol content of the films was calculated as mg/cm² for each disc (Table 1).

**In vitro drug release from the films**

In vitro drug release studies were evaluated on the F4-F7 films which were prepared with Eudragit E 100: NE 40D polymers (100:0, 70:30, 60:40, 50:50% w/w) by using methanol:acetone solvent mixture. These group of formulations (F4-F8) were more homogeneous than the films prepared by 2-propanol (F1-F3). This was the criterium of choice in studying the in vitro release of atenolol within the F4-F7 films. Only the F8 film in this group could not be evaluated for release of atenolol, as it was very tacky to study.

Vertical Franz diffusion cells with a 20 mL receptor capacity and 2.27 cm² surface area were used in the studies. 100 mM HEPES buffer (pH 7.4) was used as a receptor medium and stirred at 600 rpm, 37 ± 0.5°C. Films were applied between the chambers with cellulose acetate membranes (pore size of 0.22 mm), the released amounts of atenolol were quantitated by collecting 0.5 mL samples at 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, 54, 60, 72, 78 h and analyzed spectrophotometrically at 274 nm. The results of drug released at the end of 78 h period and release profiles are given in Figure 1, respectively.
In vitro and in vivo transdermal studies of atenolol using iontophoresis

The in vitro release rates for atenolol were evaluated by linear regression analysis and statistical parameters for drug release were evaluated by paired Student-t test using GraphPad, Instat 3.0. The parameters of the mathematical models and descriptive statistics of regression for the release data of the films F4-F7 were evaluated by analysis of variance (ANOVA) using Microsoft, SPSS 9.0 programme (Table 2).

Validation of spectrophotometric analysis for determination of atenolol in HEPES buffer was done by performing linearity and range, precision, accuracy and specificity according to ICH Guidelines Q2R1 (20). Accuracy and precision of the method was tested six times from the 0.1 ng/mL concentration level of the calibration curve. Result of 104.5 ± 0.363% indicated accuracy and paired t-test was applied on the data of two consecutive days to show

Table 1. The composition (% w/w) and the characterization of the films.

<table>
<thead>
<tr>
<th>Film Code</th>
<th>Eudragit E100:NE40D Ratio</th>
<th>Acetone:2-propanol (mL)</th>
<th>Acetone:methanol (mL)</th>
<th>Drug amount (mg/cm²)*</th>
<th>Thickness (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>70:30</td>
<td>3:2</td>
<td>-</td>
<td>1.27 ± 12.5</td>
<td>0.330 ± 8.66</td>
</tr>
<tr>
<td>F2</td>
<td>60:40</td>
<td>3:2</td>
<td>-</td>
<td>1.21 ± 2.86</td>
<td>0.377 ± 0.29</td>
</tr>
<tr>
<td>F3</td>
<td>50:50</td>
<td>3:2</td>
<td>-</td>
<td>1.31 ± 10.4</td>
<td>0.355 ± 2.29</td>
</tr>
<tr>
<td>F4</td>
<td>100:0</td>
<td>-</td>
<td>4:1</td>
<td>1.28 ± 9.84</td>
<td>0.340 ± 1.73</td>
</tr>
<tr>
<td>F5</td>
<td>70:30</td>
<td>-</td>
<td>4:1</td>
<td>1.34 ± 4.16</td>
<td>0.343 ± 0.58</td>
</tr>
<tr>
<td>F6</td>
<td>60:40</td>
<td>-</td>
<td>4:1</td>
<td>1.35 ± 4.58</td>
<td>0.332 ± 1.04</td>
</tr>
<tr>
<td>F7</td>
<td>50:50</td>
<td>-</td>
<td>4:1</td>
<td>1.48 ± 13.1</td>
<td>0.330 ± 1.00</td>
</tr>
<tr>
<td>F8</td>
<td>0:100</td>
<td>-</td>
<td>4:1</td>
<td>0.89 ± 22.7</td>
<td>0.293 ± 1.15</td>
</tr>
</tbody>
</table>

*All values for drug amount and thickness are expressed as the mean ± SD×10⁻² (n =3).

Table 2. Parameters of the mathematical models and descriptive statistics of regression for the release data of the films F4-F7.

<table>
<thead>
<tr>
<th>Model</th>
<th>Statistics</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero order</td>
<td>r²</td>
<td>0.877</td>
<td>0.938</td>
<td>0.940</td>
<td>0.961</td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>9.53 × 10⁻³</td>
<td>1.63 × 10⁻²</td>
<td>1.65 × 10⁻²</td>
<td>2.04 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>RMS</td>
<td>2.11 × 10⁻²</td>
<td>2.93 × 10⁻²</td>
<td>2.86 × 10⁻²</td>
<td>2.81 × 10⁻²</td>
</tr>
<tr>
<td>First order</td>
<td>r²</td>
<td>0.939</td>
<td>0.889</td>
<td>0.865</td>
<td>0.849</td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>1.29 × 10⁻²</td>
<td>1.98 × 10⁻²</td>
<td>1.96 × 10⁻²</td>
<td>2.59 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>RMS</td>
<td>8.21 × 10⁻³</td>
<td>3.74 × 10⁻³</td>
<td>4.57 × 10⁻³</td>
<td>9.04 × 10⁻³</td>
</tr>
<tr>
<td>Higuchi</td>
<td>r²</td>
<td>0.977</td>
<td>0.998</td>
<td>0.995</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>7.27 × 10⁻³</td>
<td>0.122</td>
<td>0.122</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td>RMS</td>
<td>3.99 × 10⁻³</td>
<td>1.06 × 10⁻³</td>
<td>2.22 × 10⁻³</td>
<td>6.66 × 10⁻³</td>
</tr>
</tbody>
</table>

r², determination coefficient; k, dissolution rate constant; RMS, residual mean square

Table 3. Mean plasma and skin concentrations of atenolol after the application of patches (F5), in vivo (n=5).

<table>
<thead>
<tr>
<th>Application</th>
<th>C_plasma (µg/ml) ± SE</th>
<th>C_skin (µg/cm²) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 min (without iontophoresis)</td>
<td>&lt; LOQ</td>
<td>3.10 ± 0.355</td>
</tr>
<tr>
<td>90 min (with iontophoresis)</td>
<td>0.399 ± 0.0450</td>
<td>1.81 ± 0.026</td>
</tr>
<tr>
<td>180 min (without iontophoresis)</td>
<td>0.103 ± 0.0150</td>
<td>0.775 ± 0.346</td>
</tr>
<tr>
<td>180 min (with iontophoresis)</td>
<td>0.510 ± 0.0560</td>
<td>2.27 ± 0.374</td>
</tr>
<tr>
<td>90 min (with oleic acid)</td>
<td>0.825 ± 0.336</td>
<td>2.5 ± 2.52</td>
</tr>
</tbody>
</table>

Validation of spectrophotometric analysis for determination of atenolol in HEPES buffer was done by performing linearity and range, precision, accuracy and specificity according to ICH Guidelines Q2R1 (20). Accuracy and precision of the method was tested six times from the 0.1 ng/mL concentration level of the calibration curve. Result of 104.5 ± 0.363% indicated accuracy and paired t-test was applied on the data of two consecutive days to show
intermediate precision \((p > 0.05)\). Also, the low values of standard error of the slope \((4.563 \pm 0.016)\) and good correlation coefficient values \((1.000)\) establish the linearity of the method between \(0.01-0.25\) mg/mL concentration ranges. Specificity of the method was studied with the existence of plasticizer and polymers.

**In vitro drug release by iontophoresis**

In order to show the effect of iontophoresis on the *in vitro* release rate of atenolol, direct current densities of \(0.1\) mA/cm\(^2\) and \(0.5\) mA/cm\(^2\) were applied on the formulations continuously for 6 h in comparison with the control group. Ag/AgCl electrodes attached to a constant current source were placed to vertical Franz cell in order to ensure the current. 100 mM HEPES buffer (pH 7.4) was used as a medium, extraneous ions were not used with the buffer and samples were analyzed spectrophotometrically at 274 nm. The release rates of atenolol from the films are given in Figures 2 and 3.

**In vivo application of the patches**

Male Wistar rats (250-300 g) were used in the studies. The animals were housed under standard laboratory conditions at room temperature with 60% relative humidity and supplied with normal pellet diet and water *ad libitum*. All experiments had previously been approved by the Local Ethic Committee. The rats were anesthetized by ketamine \((75\) mg/kg, *ip*) before the films (1 cm\(^2\)) were transdermally applied to chest region \((n = 5)\). The hair was removed by electrical clippers and the patches

Figure 1. *In vitro* release profiles of atenolol from F4-F7 formulations, \((n = 3)\). Significant difference was found between F4-F7, F5-F7 and F6-F7 \((p < 0.05)\). Significant difference was not found between F5-F6 \((p > 0.05)\).

Figure 2. The amount of atenolol released from F4-F7 formulations \((n = 3)\) \((a: \text{Control}, b: 0.1\) mA/cm\(^2\) and c: 0.5 mA/cm\(^2\) current application).
In vitro and in vivo transdermal studies of atenolol using iontophoresis

were applied to the skin with the aid of hypoallergenic knitted tape.

In comparison with passive diffusion, anodal iontophoresis was performed to modify the release of atenolol from the film F5 (Eudragit E 100:NE 40D, 70:30% w/w) for 90 and 180 min. The system with a constant current source to achieve direct current density of 0.5 mA/cm² and Ag/AgCl electrodes were used to provide electrical flow. The receptor electrode was embedded into a gauze pad filled with HEPES buffer.

Chemical enhancer – oleic acid, was also used as an alternative penetration enhancement method to iontophoresis. Oleic acid 2.5% w/v in ethanol:water (1:3 v/v) was pre-applied for 30 min before the experiments with the help of a gauze pad loaded with 2 mL of enhancer solution, the skin was washed and air-exposed for 30 min before the films were placed (6).

After iontophoresis or oleic acid treatment, collected blood (1 mL) samples were centrifuged at 3000 rpm for 10 min (Beckman, USA) and the plasma samples were stored at -80°C. The whole skin was excised from the corresponding area of the application site (1 cm²) and atenolol was extracted from the skin by using acetonitrile:serum physiological solution (1:1 v/v), (21). The mean plasma and skin concentrations of atenolol can be seen in Table 3.

The atenolol concentrations in plasma and skin were determined by HPLC (Shimadzu, Japan) analysis under following conditions: LC-10AD pump, SIL-10AXL autoinjector, SGE SS Wakosil II, 5 C18 RS 5 mm (250 × 4.6 mm) column with CTO-10A column oven, fluorescence detector (RF10AXL), FCV10AL Low Pressure Gradient Mixer, acetonitrile:methanol:water (40/25/35 v/v/v) as mobile phase, sotalol as an internal standard (500 mg/mL), λex:275 nm and λem:300 nm as detector wavelengths, 2 mL/min flow rate, 20 mL injection volume and the retention time was found as 27 min for S-enantiomer. In order to determine atenolol enantiomers from plasma or skin, a derivatization step was carried out to obtain carbamate derivatives of atenolol. MCF (0.187 M) was used as a chiral derivatization reagent in a medium of pH 9 with 200 mL saturated sodium bicarbonate solution, as reported by Mehrvar et al. (22, 23).

Analytical validation of HPLC analysis for quantitation of atenolol in the medium was done by performing linearity and range, precision, accuracy and specificity according to ICH Guidelines Q2R1 and SFSTP guidelines (20, 24) and the results were expressed as the mean values ± SE or RSD %. Paired Student’s t test was used to compare the data for the intermediate precision with a confidence interval of 95% and the p > 0.05 values were considered to be statistically not significant. Selectivity of the method was examined in relation to interference from the additive ketamine which is used to anesthetize rats in clinical studies. The results are given in Table 4 and Figure 4.

In order to show the stability of atenolol in plasma spiked samples, drug solutions were kept at room temperature for 24 h and no change was seen in the chromatographic conditions or absorbance readings.

RESULTS AND DISCUSSION

It has been demonstrated that Eudragit E 100 can be used as a self-adhesive matrix material in dermal and transdermal therapy by combining with excipients (14, 25). In this study, films prepared with Eudragit E 100 (F4) were found brittle, so Eudragit NE 40D was incorporated to the films at different ratios in order to ensure flexibility; howev-
er, the formulation prepared without Eudragit E 100 (F8) was more tackier than expected.

It was reported (26) that there would be variation between the thickness of the films because of separate preparation and this would affect in vitro release data. Low standard deviations for thickness indicate a good reproducibility in the production of formulations (19). Appropriate choice of solvents can also affect film thickness and drug contents. Relatively high standard deviations were obtained in thickness and drug content of the formulations prepared by 2-propanol (F1-F3) because of slow evaporation (27). This indicates a relatively poorer reproducibility than for the formulations prepared by methanol (F4-F8). Thus, methanol:acetone mixture was preferred in the preparation of the films (Table 1).

A plasticizer type and amount was also important at the preparation. The amount of plasticizer has importance also for drug release. With increasing amount of plasticizer, higher drug diffusion rates
can be achieved (26). As a result of pre-studies, triacetin was chosen as a plasticizer at the concentration of 20% w/w, which was reported to have a tackifier effect on formulations as well as performing good plasticizer effect with Eudragit E 100 and NE 30D (28, 29).

Evaluating the variation between the batches, the effect of polymer type and ratio on the in vitro release of atenolol was studied on four formulations (F4-F7). The release of atenolol from these films was slower with regard to increase in Eudragit E 100 content. This could be attributed to basic nature of atenolol with a pKa of 9.5 (30-32). It was observed that the release rate of atenolol was significantly increased by decreasing the ratio of Eudragit E100 from 100% w/w (F4; 0.646 ± 0.0722 mg/cm²) to 50% w/w (F7; 1.30 ± 0.0867 mg/cm²) at the end of 78 h period. This could be also attributed to the increasing ratio of Eudragit NE 40D (14). However, the release profiles of F5 (1.06 ± 0.0250 mg/cm²) and F6 (1.09 ± 0.0433 mg/cm²) formulations were not found different statistically (p > 0.05) (Figure 1). The kinetic results were best fitted to Higuchi release kinetics which indicates that the transport of the drug from the films was governed by a diffusion mechanism (Table 2).

In order to show the effect of iontophoresis, 0.1 and 0.5 mA/cm² direct current densities were applied for 6 h to F4-F7 films. The results for the differences between slope of control and treatment groups were compared statistically using paired Student-t test. An increase in the amount of atenolol released was found significant (p < 0.05) for 0.5 mA/cm² current application, however the amount of atenolol released with the use of 0.1 mA/cm² current was not found significant in comparison with the control group (Figures 2 and 3).

The studies have shown that iontophoresis, at current densities greater than 0.3 mA/cm² can electroporate the skin to a certain degree, by causing the formation of transient aqueous pores by electroporating the skin, and that these pores constituted a significant transport route during iontophoresis (9). On the other hand, oleic acid is a fatty acid highly ionizable at physiological pH, increases lipid fluidity and thus enhances water permeability. It was reported that oleic acid is effective with atenolol at a concentration of 1% w/v (33).

In this study, 0.5 mA/cm² direct current was used in comparison with 2.5% w/v oleic acid to enhance the permeation of drug from the skin, in vivo. As a result of the in vivo study, without any enhancement mechanism plasma concentration of atenolol was under LOQ of the method of analysis (50 ng/mL) for 90 min and the amount of atenolol in the plasma was higher in the presence of oleic acid than by iontophoresis (Table 3). When the concentrations in the skin are compared, it seems to be a nearly 7 fold increase in the amount of atenolol with oleic acid than iontophoresis. A higher skin concentration of atenolol with oleic acid (12.5 mg/cm²) resides to modulation of the extracellular lipid domain in stratum corneum (6, 34). In contrast, 1.81 mg/cm² of skin concentration by iontophoretic delivery can be assumed to a transappendageal pathway of the skin (8). Higher standard deviations for oleic acid treatment than iontophoresis application (Table 3) could result from the great biological variations among the animals by chemical enhancers (35). The detected plasma concentration of atenolol for the application of 90 min iontophoresis (0.399 ± 0.045 mg/mL) increased to 0.510 ± 0.056 mg/mL by increasing the duration time to 180 min. These lower biological variations and an increase in the amount of atenolol by direct current of 0.5 mA/Cm² application could indicate that the drug delivered was proportional to the applied current and duration of iontophoresis (8).

CONCLUSION

The present study shows that iontophoresis is a useful method to enhance the transdermal permeation of hydrophilic atenolol from Eudragit films. Although oleic acid effectively enhanced the permeation of atenolol from skin, high concentration of chemical enhancer was used to achieve this result. Due to the mechanism of iontophoresis, it could be proposed as an alternative method to chemical enhancers.

Acknowledgments

Eudragit polymers (E100, NE40D) (ROEHM Pharma, Germany) were kindly donated from Karadeniz Pharmaceutical Warehouse (Istanbul, Turkey).

REFERENCES


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