Design, *In Vitro* Stability, and Ocular Hypotensive Activity of *t*-Butalone Chemical Delivery Systems

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ABSTRACT: The objective of this work was to synthesize two bioreversible diacyl derivatives of *t*-butalone (chemical delivery systems), determine their chemical and *in vitro* stability, and investigate their potential use as topical antiglaucoma agents. The stability of these compounds was determined in isotonic phosphate buffers (pH range 5–8) and in selected biological media, including human whole blood, rabbit and rat blood, and the anterior segment tissues of rabbit. The ocular hypotensive activity of these compounds in unrestrained, normotensive albino rabbits was determined with a pneumatonometer. The two compounds were stable at lower pH. The stability decreased as the pH increased, suggesting their lability to base-catalyzed hydrolysis. These compounds exhibited significant differences in the hydrolytic rates in the whole blood among species examined (rat > rabbit > human). The observed rates of disappearance in different ocular tissues were indicative of relative enzyme activity in these media (iris-ciliary body > cornea > aqueous humor). The two compounds exhibited a significant ocular hypotensive activity ($P < 0.01$) at 2% dose level. The peak activity was found between 2 and 4 h, and the activity was maintained for 4.5 to 7 h. The dipivalyl derivative of *t*-butalone exhibited more pronounced decrease in intraocular pressure than that of diisovaleryl derivative. The present study suggests the possible use of diacyl derivatives of *t*-butalone as ocular hypotensive agents. © 2001 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 90:1026–1033, 2001

Keywords: chemical delivery systems; stability; *t*-butalone esters; antiglaucoma agents

INTRODUCTION

Design and development of topical ocular delivery systems is one of the most interesting and challenging areas of current research. Medications designed for ophthalmic use are currently undergoing a process of optimization because of inherent problems associated with absorption efficiencies of topically applied drugs. The poor ocular availability of topical ophthalmic agents is attributed to the physiological protective mechanisms of the eye, such as instilled volume drainage and tear turnover, the biological (corneal) barrier, and the inherent physicochemical insufficiencies of the drug molecule itself.[1,2]

In recent years, novel metabolism-based drug delivery concepts and strategies were proposed to design and develop safe, specific, and effective ophthalmic agents.[3–9] One of the popular concepts is the site-specific chemical delivery system (CDS) approach that is based on the predictable multistep metabolic activation of bioreversible, inactive agents at the site of action.[3,5,10] The chemical delivery system concept was successfully applied to design and develop site-specific, short-acting mydriatic[9,11–13] and anti-glaucoma agents.[14–16] The present work expands the applicability of the CDS concept to design biorever-

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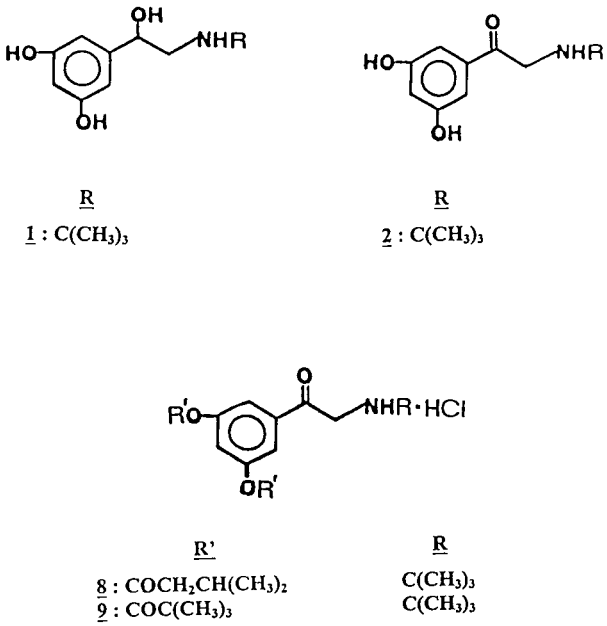

Figure 1. Chemical structures of *t*-butaline (**1**), *t*-butalone (**2**), and diacyl ketone derivatives (chemical delivery systems; **8** and **9**).

sible *t*-butalone derivatives to target *t*-butaline (**1**) to iris-ciliary body tissues in the eye. According to this concept, lipophilic ester derivatives (**8**, **9**) of the inactive chemical precursor of **1**, the corresponding ketone (**2**) is capable of site-specific delivery of the active **1** (Figure 1), via a reduction-hydrolysis sequence, as shown in the case of diacyl adrenalone derivatives.[11]

t-Butaline (**1**) is selective β_2-adrenoreceptor agonist and is used in the treatment of bronchoconstrictive disorders. Some selective β_2-receptor agonists, such as *t*-butaline, salbutamol, and carbuterol, were found to lower intraocular pressure (IOP) as effectively as isoproterenol in rabbits.[17] Their potential use as ocular hypotensive agents is based on the suggestion that IOP could be lowered effectively with minimal cardiac stimulation, which is a β_1 -adrenoreceptor effect. However, by virtue of hydrophilic nature of **1**, topical administration of their aqueous solutions at 2% dose level had little effect on IOP of normotensive rabbits.[17,18] This result may be attributed to their poor permeability across biphasic corneal membrane that is due to their hydrophilic nature.

In the present study, chemical modifications of *t*-butaline are proposed to enhance its corneal permeability and to assess the validity of CDS concept in delivering *t*-butalone derivatives site-specifically to iris-ciliary body tissues. The chemical and *in vitro* stability of novel bioreversible derivatives were investigated in buffers (pH 5–8) and in selected biological test media. The ocular hypotensive activity was evaluated in the rabbit model.

MATERIALS AND METHODS

All chemicals used were of reagent grade. Reagents and deuterated solvents were obtained from Aldrich Chemical Company unless otherwise stated and were used without further purification. Melting points were determined with a Fisher Johns melting point apparatus and were uncorrected. The proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with Varian T-90 or VXR-300 spectrometers. Chemical shifts are reported as parts per million (ppm) units relative to tetramethylsilane (TMS) as internal standard. Elemental analysis was performed by Atlantic Microlab Inc. (Atlanta, GA).

Synthesis

The synthetic scheme of diacyl derivatives of *t*-butalone is depicted in Figure 2.

Synthesis of 3′,5′-Diisovaleryloxyacetophenone, 4

To a continuously stirred solution of 3′,5′-dihydroxyacetophenone, **3** (10 g) in pyridine (50 mL) was added isovaleryl chloride (7.6 g) at ice-cooling temperature. The reaction mixture was stirred overnight at room temperature and then poured into ice water (100 mL). The oil was extracted twice with ethyl acetate (100 mL each time), and the extract was washed first with water followed by 10% aqueous HCI and water. The extract was dried over anhydrous Na_2SO_4, and the solvent was removed by evaporation under vacuum to give **4** (13 g, 65%) as crude oil, which was then purified by column chromatography on silica gel (eluent CH_2Cl_2). ^{1}H NMR ($CDC1_3$): 1.13 (12H, d, $J = 9$ Hz, CH_3), 7.13 (1H, t, $J = 2$ Hz, C_4H), 7.53 (2H, d, $J = 2$ Hz, $C_{2'}-$ and $C_{6'}-H$).

Synthesis of 3′,5′-Dipivaloyloxyacetophenone, 5

To a continuously stirred solution of 3′,5′-dihydroxyacetophenone, **3** (4.2 g) in pyridine (20 mL) was added pivaloyl chloride (4.6 g) at ice-cold

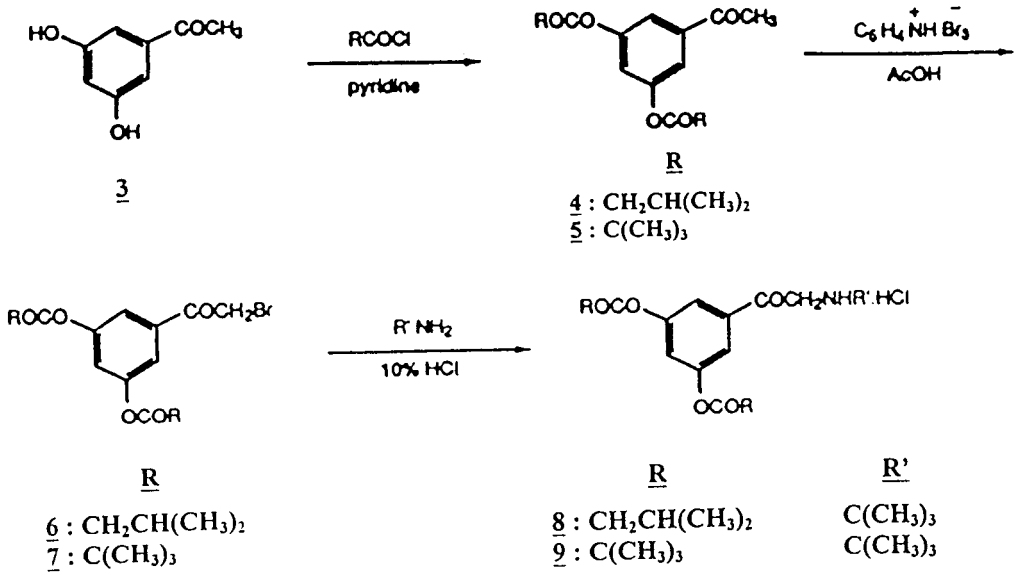

Figure 2. Synthetic reaction sequence of diacyl ketone derivatives (chemical delivery systems) of *t*-butaline (**8**) and (**9**).

temperature. The reaction mixture was stirred for 5 h at room temperature and then poured into ice-water (50 mL), which was later extracted twice with ethyl acetate, taking 100 mL each time. The extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated under vacuum to give the residue. The crude products were purified by silica gel column chromatography (eluent CH_2Cl_2) to obtain **5** (7.0 g, 77%) as colorless oil. ^{1}H NMR ($CDC1_3$): 1.4 (s, 18H, $CH_3 \times 6$), 2.6 (3H, s, $COCH_3$) 7.05 (1H, t, $J = 2$ Hz, 4′-H), 2.46 (2H, d, $J = 2$ Hz, 2′ and 6′-H).

Synthesis of 2-Bromo-3′,5′-diisovaleryloxyacetophenone, 6

To a continuously stirred solution of **4** (13 g) in AcOH (50 mL) was added pyridiniurn bromide perbromide (15 g). The mixture was stirred for about an hour at room temperature and then poured into ice water (200 mL). After extraction with ethylacetate (200 mL), the extract was washed with water and aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4, and then evaporated in vacuum to give a crude oil (14.8 g). This material was later purified by column chromatography on silica gel using CH_2Cl_2 as eluent to obtain **6** (11. 8 g, 74%) as oil. ^{1}H NMR ($CDCl_3$): 1.10 (12H, d, $J = 9$ Hz, $CH_3 \times 4$), 1.80–2.46 (2H, m, $CH \times 2$), 2.40 (4H, s, $CH_2 \times 2$), 4.40 (2H, s, $-CH_2Br$), 7.15 (1 H, t, $J = 2$ Hz, C_4-H), 7.53 (211, d, $J = 2$ Hz, C_2- and C_6-H).

Synthesis of 2-Bromo-3,5′-dipivaloyloxyacetophenone, 7

To a continuously stirred solution of **5** (7.0 g) in AcOH (70 mL) was added pyridinium bromide perbromide (15.5 g). After stirring for about an hour at room temperature, the mixture was poured into ice water (200 mL) and extracted with AcOEt (200 mL). The extract was washed with water, dried over anhydrous Na_2SO_4, and then evaporated in vacuum to give the crude **9**, which was then recrystallized from hexane to obtain **7** (6.0 g, 67%) as colorless needles. The melting point was 125–127°C. ^{1}H NMR ($CDC1_3$): 1.40 (18H, s, $CH_3 \times 6$), 4.40 (2H, s, $COCH_2Br$), 7.03 (1H, $J = 2$ Hz, 4′-H), 7.43 (2H, d, $J = 2$ H, 2′ and 6′-H).

Synthesis of 2-tert-Butylamino-3′,5′-diisovaleryloxyacetophenone, 8

To a continuously stirred solution of tertiary butylamine (3.2 g) in CH_2Cl_2 (40 mL) was added a solution of **6** (3.2 g in 10 mL of CH_2Cl_2) under ice-cold conditions. After stirring for about an hour, CH_2Cl_2 (40 mL) and 10% aqueous HCI (50 mL) were added while stirring. The organic layer was separated, dried over anhydrous Na_2SO_4, and then evaporated under vacuum. The residue thus obtained was recrystallized from a mixture of CH_2Cl_2 and ether that contained few drops of water to obtain hydrochloride salt of **8** (0.81 g, 24%) as colorless needles. The melting

point was 186–188°C. ^{1}H NMR ($CDC1_3$): 1.10 (12H, d, $J = 6$ Hz, $CH_3 \times 4$), 1.50 (9H, s, $CH_3 \times 3$), 1.85–2.26 (2H, m, $CH(CH_3)_2 \times 2$), 2.46 (4H, s, $CH_2 \times 2$), 4.60 (2H, s, $-C-CH_2-N$), 7.20 (2H, t, $J = 1.5$ Hz, C_4-H), 7.50 (2H, d, $J = 1.5$ Hz, C_2- and C_6-H), 9.50 (IH, bs, NH).

Synthesis of 2-tert-Butylamino-3′,5′-dipivaloyloxyacetophenone, 9

To a continuously stirred solution of tertiary butylamine (4.0 g) in CH_2Cl_2 (50 mL) was added **7** (4.0 g) under ice-cooling conditions. After stirring for about an hour, CH_2Cl_2 (50 mL) and 10% aqueous HCI (50 mL) were added to the mixture. The organic layer was separated, dried over anhydrous Na_2SO_4, and evaporated under vacuum to give **9**. HCl was washed with Et_2O followed by recrystallization from $CH_2Cl_2-Et_2O$ to give 13.HCl (1.3 g, 30%). The melting point was 196°C. ^{1}H NMR ($CDC1_3$): 1.40 (18H, s, $CH_3 \times 6$), 1.55 (9H, s, $CH_3 \times 3$), 4.50 (2H, d, $J = 1.5$ Hz), 7.10 (1H, t, $J = 3$ Hz), 7.56 (2H, d, $J = 3$ Hz), 9.50 (1H, bs, NH).

Analytical Method

High-performance liquid chromatographic (HPLC) analyses of CDS (**8** and **9**) as well as the parent species (**1**) were performed with a Waters Associates multicomponent system consisting of a model 6000A double-piston reciprocating chromatography pump; model U6K universal injector; and model 440 absorbance detector, which can be operated at two different wavelengths. A 25-cm reversed-phase analytical C_{18} column (Water Corp., Milford, MA) with mean particle diameter of 5 μm was used at ambient temperature. A guard column (Rainin Instrument Inc., Woburn, MA) was connected to protect the analytical column from contamination of fine particles of sizes ≥ 0.5 μm present in biological materials. The guard column has a 3-cm cartridge (RP-18/18-GU) (Brownee Labs) which had a 4.6-mm internal diameter and a 10-μm sorbent packing. The mobile phase consisted of 50% acetonitrile, 50% potassium dihydrogen phosphate monobasic (0.05 M), and tetrabutylammonium phosphate (0.005 M). The flow rate was set at 2 mL/min. With absorbance detection at 254 nm, the peak heights of CDS and their corresponding parent species were monitored. The retention times for **8**, **9**, and *t*-butaline were 4.7, 4.9, and 3.5 min, respectively. The calibration curves were constructed from linear plots of peak versus concentration.

Stability in Aqueous Buffers

A stability study of **8** and **9** in isotonic phosphate buffers (0.05 M), with pH values of 5.00, 5.4, 5.8, 6.44, 6.86, 7.40, and 8.00, was performed at 37°C to determine the hydrolytic stability of the compounds under investigation as a function of pH. Ten milliliters of buffer was equilibrated at 37°C for 15 min prior to the introduction of solutions of **8** or **9** in methanol to result an initial concentration of 100 μM. The buffer was mixed by vortex for 15 s. Twenty-microliter samples were periodically withdrawn at set time intervals from the media and injected directly into the HPLC system. The disappearance of **8** or **9** was monitored by measuring the peak heights. The pseudo-first-order rate constant for disappearance of compounds under investigation was determined by linear regression from the plot of natural logarithm of peak height versus time. The pH profiles for each compound were generated by plotting apparent first-order rate constant ($\log k_{app}$) versus pH.

In Vitro Stability in Biological Media

Whole Blood

The stability of **8** and **9** was determined in human whole blood and in the whole blood of rabbits and rats. Freshly drawn heparinized human blood, obtained from the Civitan Regional Blood Center, Inc. (Gainesville, FL) was stored in a refrigerator and used within 72 h from the time it was collected. Freshly prepared solutions of **8** or **9** in methanol (50 μL, 50 μM) were mixed with 5 mL of blood that had been previously equilibrated for 10 min at 37°C in a waterbath. These preparations were then mixed thoroughly with a vortex mixer for 15 s. At set time intervals, 0.4-mL samples were withdrawn from the test medium and mixed immediately with 0.8 mL of ice-cold acetonitrile containing 10% dimethylsulfoxide (DMSO) and mixed by vortex for 15 s. The mixtures were centrifuged at 3000 *g* for 5 min. Aliquots from the supernatant were filtered using 0.45-μm HV Millipore filters and analyzed by injecting onto the HPLC column. The apparent first-order rate constants for the hydrolysis of **8** or **9** were determined by monitoring the disappear-

ance of the respective compounds by linear regression from the plot of natural logarithm of the HPLC peak height versus time. Half-lives, correlation coefficients, and rate constants were calculated for each compound studied.

The rabbit and rat blood was collected from male New Zealand albino rabbits and Sprague-Dawley rats, respectively. The blood was collected into heparinized test tubes, and these heparinized blood samples were pooled (from three animals in each case) and refrigerated. Sampling and analysis to determine hydrolytic stability of **8** and **9** in rabbit and rat blood were done according to the procedure already described.

Anterior Segment Tissues of Rabbits

Ocular tissues were obtained from male New Zealand albino rabbits weighing 2–3 kg. Rabbits were sacrificed with lethal dose of pentobarbital (100 mg/kg) administered intravenously (iv) into the marginal ear vein. Each eye was enucleated and rinsed in cold saline to remove any traces of blood. Aqueous humor was then obtained by making a single puncture at the limbus using 25 G × 5/8′ needle attached to a 3-cc syringe. Once the aqueous humor was removed, corneal and iris-ciliary body tissues were separated. Ten percent homogenates of corneal and iris-ciliary body tissues were prepared in isotonic phosphate buffer, pH 7.4, using a tissuemiser (Tekmar SDT, Cincinnati, OH) for 2 min. Aqueous humor was used as such without further dilution. The homogenates of corneal and iris-ciliary body tissues and aqueous humor were taken into stoppered tubes and incubated for 15 min at 37°C. After equilibrium had been attained, 50 μL of freshly prepared solutions of **8** or **9** in methanol were added to each milliliter of the tissue homogenate and aqueous humor. Fifty-microliter samples were withdrawn at set time points, and the enzymatic activity was destroyed by adding 200 μL of acetonitrile. The disappearance of CDS was monitored by HPLC, and the kinetic parameters, such as half-life and rate constants, were determined.

Ocular Hypotensive Activity in Rabbits

IOP was measured with a pneumatonometer (Digilab model 30R), which utilizes a pneumatic sensor. Adult male New Zealand albino (unrestrained and normotensive) rabbits, weighing 2.0–3.0 kg, were used in this study. Rabbits were kept in individual cages with free access to food and water. The compounds (**8** or **9**) were administered as 2.0% w/v solution (50 μL) in buffer, pH 7.4, or saline into both eyes of a group of six rabbits. Prior to the IOP measurements, corneal anesthesia was provided by topical administration of one drop of 0.5% proparacaine (Allergan) to minimize discomfort from tonometry. Each measurement lasted 5–10 s, and a precise reading was recorded at set time points (0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h) on a chart calibrated in mmHg. The tonometer was calibrated at least twice a day, once at the beginning of experiment and once in between, using a 3-point calibration verifier (Digilab). The reported readings are the average values of six experiments (±SEM). The significance ($P < 0.01$) of the change in IOP was determined using a Student's t test. After allowing a washout period of at least 8 days, the same group of rabbits was administered 50 μL of saline or buffer without the drug into both eyes and the readings served as control.

RESULTS AND DISCUSSION

Chemical Stability

The disappearance rates of **8** and **9** in aqueous buffers with pH values in the range of 5–8 were determined to assess their chemical stability. The pH profiles generated by plotting logarithmic values of apparent first-order rate constant, log k_{app} (based on the disappearance of the compounds under investigation from the buffer media) versus pH are shown in Figure 3. The two compounds were relatively stable at lower pH values studied and the stability decreased as the pH increased. The higher stability at low pH was expected because the nucleophiles involved in the hydrolysis of these ester compounds are protonated, thereby decreasing the possibility of nucleophilic attack and the resulting hydrolysis. The dipivalyl ester derivative (**9**) was a relatively more stable diisovaleryl ester derivative of *t*-butalone (**8**) at all the pH values studied. This result can be explained on the basis of steric hindrance of the nucleophilic attack of hydroxyl ions on the bulky tertiary butyl groups in the hydrolysis, which is consistent with our earlier findings with dipivalyl ester derivative of phenylephrone.[13] At physiological pH of 7.4, the half-lives of **8** and **9** were 347±23 and 431±28 min, respectively.

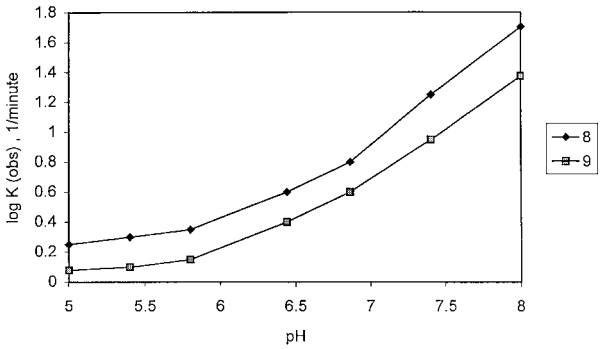

Figure 3. Experimentally determined pH–rate profiles of diacyl ketone derivatives of *t*-butaline (**8** and **9**) in pH range 5–8 at 37°C (average value of three runs was reported).

Stability in Biological Media

The *in vitro* stability of **8** and **9** was investigated in selected biological media, which include the whole blood from human, rabbit, and rat, and the anterior segment tissues and fluids of rabbit, which include 10% w/v corneal tissue homogenates, 10% w/v iris-ciliary body tissue homogenates, and aqueous humor. The half-lives based on disappearance of CDS in the biological media and in buffer solution (pH 7.4) are shown in Table 1. In all the investigations, a significant difference in the disappearance rate was found among species studied (rat > rabbit > human). The rates of disappearance of all the CDS were ~2-fold faster in rat blood than in rabbit blood, which were in turn ~2- to 3-fold greater than in human blood. The greater enzymatic activity in smaller animals, such as rats and rabbits, is well established.[19,20]

The stability data of CDS in rabbit anterior segment tissues, including corneal and iris-ciliary body homogenates and aqueous humor, indicate the following order of enzymatic activity: iris-ciliary body > cornea > aqueous humor. Compounds **8** and **9** were ~2 times more stable in corneal homogenates than in iris-ciliary body homogenates. The iris-ciliary body tissues are implicated as one of the major sites of drug metabolism because they have the most well-developed drug metabolizing systems found in the eye, so the highest rates in these tissues were expected. The half-lives of the compounds under investigation in aqueous humor were ~7–9 times longer than those in iris-ciliary body tissue homogenates.

Among the diesters tested, the diisovaleryl ester derivatives (**8**) was relatively more labile than dipivaloyl derivative (**9**) in all the biological media. The ester linkages exhibited a steric order of reactivity that had previously been reported for esterase-catalyzed hydrolysis of phenyl acetates,[21] adrenalone esters,[11] and phenylephrone esters.[13] Thus, the results suggest that it is possible to prepare bioreversible derivatives with different biological stability based on steric factors that allow the duration of action to be controlled for the desired therapeutic need.

Intraocular Pressure

The effects of single dose of **8** and **9** on the IOP of normotensive rabbits are depicted in Figure 4. The compounds were tested at 2% dose level. The change in IOP in mmHg [(treated value–treated baseline)–(control value–control baseline)] following topical administration of the CDS as a function of time is shown. The two compounds tested exhibited significant ($P < 0.01$) IOP lowering activity. The activity ($P < 0.001$) peaked between 3 to 4 h and it lasted for ~6–7 hours. Compound **9** was relatively more potent in lowering the IOP than **8** and had longer duration of action (7 h compared with ~ 6 hours for that of **8**).

Table 1. The *In Vitro* Hydrolytic Half-Lives (min) of Diisovaleryl (**8**) and Dipivalyl (**9**) *t*-Butalone of in the Biological Media and in the Buffer Solution (pH 7.4) at 37°C[a]

Biological Media	(**8**)	(**9**)
Human blood	5.42±0.4	13.80±1.5
Rabbit blood	2.41±0.2	6.0±0.9
Rat blood	1.27±0.1	3.27±0.2
Corneal tissue homogenate (10%)	3.78±0.4	8.58±0.9
Iris-ciliary body homogenate (10%)	2.23±0.1	4.66±0.3
Aqueous humor	20.38±1.8	39.29±2.9
Buffer (pH 7.4)	347.0±23	431.0±28

[a]Each half-life value presented in the table represents the mean of three experiments±SEM.

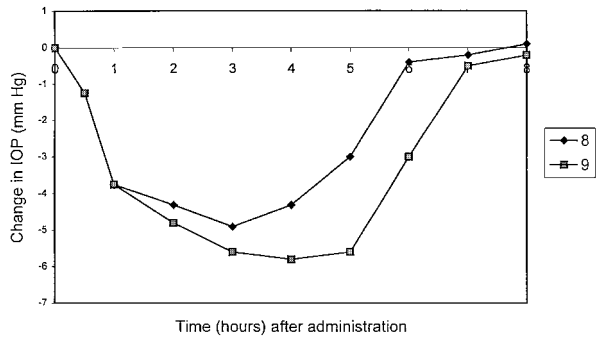

Figure 4. Effect of topical administration of a single dose of 50 μL of a 2% solution of **8** and **9** in buffer or saline on intraocular pressure (IOP) of normotensive rabbit. Each value represents average (±SEM) of six experiments.

The IOP-lowering activity of diacyl ketone derivatives of *t*-butaline, a selective β_2-receptor agonists, is not unexpected. *t*-Butaline and other selective β_2-receptor agonists, such as salbutomol and carbuterol, were shown to lower IOP of normal rabbits.[22] Furthermore, *t*-butaline was equipotent to salbutamol and the effect was found to be more than that of isoproterenol, a nonselective β-receptor agonist in the rabbit model.[22] The topical administration of aqueous solutions of *t*-butaline in normal saline (at 2% dose level), however, had little effect in lowering IOP of normotensive rabbits.[17,18] This result may be explained on the basis of their inadequate lipophilicity resulting in poor partitioning into cornea. Because the cornea consists of lipophilic epithelial and endothelial layers and a hydrophilic middle region, the collagenous stroma, biphasic solubility has been the major theory describing the transcorneal drug permeation.[23,24] Substitution of two hydroxyls on the rings of compounds **3** by ester groups renders them more lipophilic, thereby improving their penetration into cornea across epithelium. After penetrating the corneal membrane barrier, the effectiveness of CDS becomes dependent on the ability of the target tissue to regenerate the parent compounds, **1** from CDS (**8** and **9**, respectively) at an optimal rate.[15]

CONCLUSIONS

The diacyl derivatives of *t*-butalone exibited significant IOP lowering activity in normotensive rabbits, suggesting their use as potential ocular hypotensive agents. Of the two compounds investigated, the dipivaloyl derivative of *t*-butalone showed pronounced and longer duration of action. The chemical and biological stability studies of these compounds suggest that with the proper selection of stearic bulk in the esterification, the duration of action can be manipulated.

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