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In-vitro Hydrolysis, Permeability, and Ocular Uptake of Prodrugs of *N*-[4-(Benzoylamino)phenylsulfonyl]glycine, a Novel Aldose Reductase Inhibitor

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Abstract

To enhance the ocular uptake of *N*-[4-(benzoylamino)phenylsulfonyl]glycine (BAPSG), two ester (methyl and isopropyl) prodrugs were synthesized and evaluated for their stability in various buffers (pH 1–9), hydrolysis in rabbit ocular tissues (cornea, conjunctiva, iris-ciliary body, lens, aqueous humor, and vitreous humor), transport across cornea and conjunctiva, and in-vivo uptake following topical administration.

Over the pH range of 1–9, the rate constants for degradation ranged from 5·67 to $218{\cdot}9\times10^{-3}\,h^{-1}$ for the methyl ester and from 3·14 to $4{\cdot}45\times10^{-3}\,h^{-1}$ for the isopropyl ester. At all pH conditions, the isopropyl ester was more stable when compared with the methyl ester. A change in buffer concentration at pH 7·4 did not influence the stability of the prodrugs. The prodrugs were rapidly hydrolysed in the tissue homogenates, with the rate constants for hydrolysis ranging from 1·98 to $7{\cdot}2\times10^{-3}\,min^{-1}$ for the methyl ester and 3·32 to $6{\cdot}53\times10^{-3}\,min^{-1}$ for the isopropyl ester. The in-vitro permeability of the methyl ester was less than the parent drug across cornea and conjunctiva. Isopropyl ester levels were not detectable in the receiver chamber even at the end of the 4-h transport study. Following topical administration of BAPSG and the two prodrugs at a dose of 60 μg/eye, the lowest levels were seen in vitreous humor for parent compound and its methyl ester. In general, the tissue uptake of methyl ester was less than BAPSG. Isopropyl ester levels were below detection limits in all the ocular tissues.

Lipophilic ester prodrugs of BAPSG showed good aqueous solution stability in tissue homogenates. However, these prodrugs lacking the free carboxylate anion exhibited reduced in-vitro permeability and in-vivo uptake, suggesting the importance of free carboxylate anion in the delivery of BAPSG.

N-[4-(Benzoylamino)phenylsulfonyl]glycine (BAPSG; **I**, Figure 1), a selective aldose reductase inhibitor (Mayfield & DeRuiter 1987; DeRuiter et al 1987, 1991), is of potential value in preventing diabetic complications of the eye including retinopathy (Kinoshita 1965; Kador et al 1990), cataract (Hotta 1997), and corneal epitheliopathy (Datiles et al 1983). It is well established that the polyol pathway, proceeding in two steps, plays a critical role in diabetic complications (Van Heyningen 1959; Kinoshita 1990; Lee et al 1995; Hotta 1997). During hyperglycaemia, aldose reductase (alditol–$NADP^+$ oxidoreductase, EC 1.1.1.21) utilizes NADPH to reduce glucose to sorbitol, a membrane impermeable polyol that accumulates in cells, thereby leading to diabetic complications. Inhibition of aldose reductase has been shown to delay or prevent diabetic complications by reversing sorbitol accumulation and the biochemical changes associated with hyperglycaemic conditions (Lightman 1993; Yabe-Nishimura 1998). Various structurally diverse compounds obtained from synthetic and natural origin have exhibited very good in-vitro aldose reductase inhibitory activity. However, none are being marketed due to side effects associated with non-specific inhibition of

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other members of the oxido-reductase family of enzymes or due to lack of significant clinical efficacy (Spielberg et al 1991; Engerman & Kern 1993). Such lack of clinical efficacy can be partially attributed to poor ocular tissue penetration and retention of the previously tested inhibitors. The goal of our research is to identify novel aldose reductase inhibitors capable of attaining therapeutic levels in the ocular tissues. Topical administration is likely to achieve significant drug levels in the ocular tissues while allowing systemic uptake of the drug.

Among a series of compounds, BAPSG was selected for further development based on its high activity and its permeability across cornea and conjunctiva (Kompella et al 1999). The empirical indicators of ocular effectiveness and systemic effectiveness, estimated as permeability coefficient across cornea/IC50 and permeability coefficient across conjunctiva/IC50, were high for BAPSG compared with other compounds tested. Furthermore, BAPSG displays concentration-dependent enzyme kinetic profiles similar to well-established aldose reductase inhibitors such as sorbinil and tolrestat (DeRuiter & Mayfield 1990). Competitive and multiple enzyme inhibition studies have indicated that BAPSG has unique aldose reductase binding sites and that it selectively inhibits aldose reductase (Davis et al 1993).

Following topical administration, less than 10% of ocular drugs such as pilocarpine and flurbiprofen reached intraocular tissues (Lee & Robinson 1986). The bioavailability of a topically administered drug depends on the physicochemical properties of the drug including its partition coefficient (log P) and degree of ionization (pK_a). The pK_a, log P and IC50 of BAPSG are 3·35, 1·09 and 0·4 μM, respectively. The apparent permeability coefficient of BAPSG across cornea and conjunctiva was $7{\cdot}44 \pm 1{\cdot}49$ ($\times 10^{-6}$ cm s^{-1}) and $15{\cdot}79 \pm 0{\cdot}27$ ($\times 10^{-6}$ cm s^{-1}), respectively (Kompella et al 1999). BAPSG contains a free carboxylic acid group that can be esterified to form lipophilic prodrugs. Lipophilic prodrugs are bioreversible and are designated to improve drug absorption through increased partition coefficient (Schoenwald & Ward 1978; Bundgaard et al 1986a). The prodrug approach has already been used to improve the ocular absorption of various drugs including adrenaline (Bodor & Visor 1984), pilocarpine (Bundgaard et al 1985, 1986b), prostaglandins (Bito 1984), timolol (Chang et al 1988), and acyclovir (Hughes & Mitra 1993). Such lipophilic prodrugs can reduce the topical dose or dosing frequency, and hence, the risk of side effects.

We have tested the utility of lipophilic prodrugs in enhancing BAPSG delivery to the eye. Methyl and isopropyl ester prodrugs of BAPSG (methyl-BAPSG (**II**) and isopropyl-BAPSG (**III**)) were synthesized (Figure 1). Stability in aqueous solutions and ocular tissue homogenates, corneal and conjunctival permeability, and in-vivo uptake of these prodrugs were assessed.

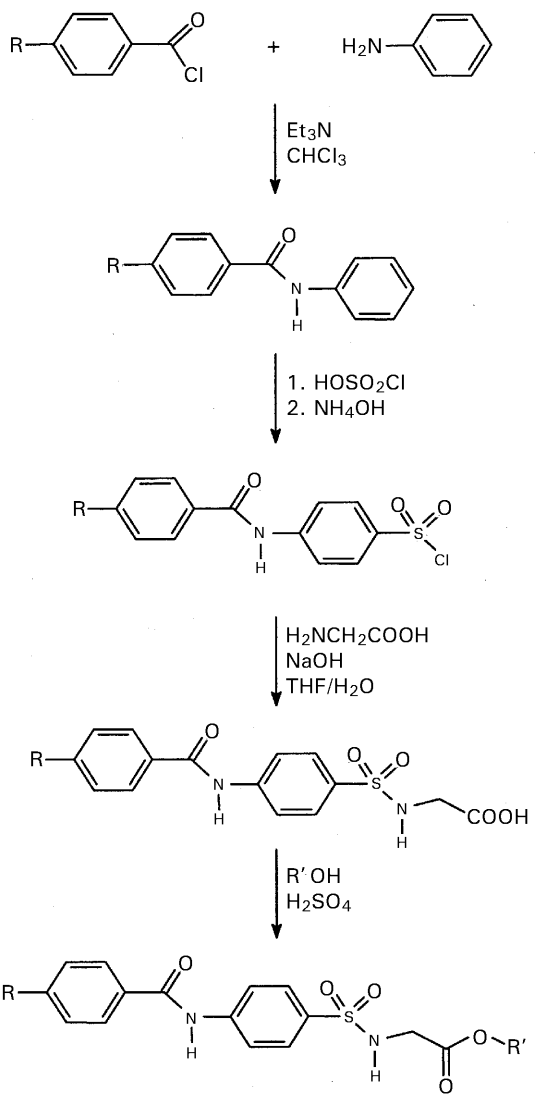

Figure 1. Scheme for the synthesis of ester prodrugs of BAPSG.

Materials and Methods

Materials

HPLC grade acetonitrile, methanol and glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ). Hydroxypropyl-β-cylclodextrin (HP-β-CyD) was a gift from American Maize-Products Co. (Hammond, IN). Other chemicals

used for preparing buffer solutions and formulations were obtained from Sigma Chemical Co. (St Louis, MO). In-vitro and enzyme hydrolysis studies were conducted using a Dubnoff metabolic shake incubator (Precision Scientific Co., Chicago, IL). Male New Zealand White rabbits (2·5–3·5 kg) were obtained from Knapp Creek Rabbitry (Amana, IA). All animals were handled in accordance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23).

Synthesis of BAPSG prodrugs

The general scheme for the synthesis of ester prodrugs of BAPSG is shown in Figure 1. The procedure for each ester is described below.

*Synthesis of methyl-BAPSG (**II**).* A solution of 470 mg BAPSG (synthesized as reported by Mayfield & DeRuiter (1987)) in 25 mL methanol with one drop of concentrated sulphuric acid was heated at reflux for three days. The solvent was removed under reduced pressure and the resulting product suspended in water, filtered, and washed with water (2×10 mL). The crude product was air-dried and recrystallized from a minimum amount of boiling methanol. The purified product was isolated by filtration and oven-dried under low heat (>100°C). The structure and purity of the product was confirmed by proton NMR (methyl singlet integrating for 3 protons at 3·85 ppm), mass spectrometry (MW 348) and elemental analysis (theoretical: C, 55·16, H, 4·63, N, 8·04; found: C, 55·16, H, 4·69, N, 8·03). The melting point of methyl-BAPSG was 202–203°C.

*Synthesis of isopropyl-BAPSG (**III**).* A solution of 500 mg BAPSG in 40 mL isopropyl alcohol with one drop of concentrated sulphuric acid was heated at reflux for three days. The solvent was removed under reduced pressure and the resulting product suspended in water, filtered and washed with water (2×10 mL). The crude product was air-dried and recrystallized from a minimum amount of boiling isopropyl alcohol. The purified product was isolated by filtration and oven-dried under low heat (>100°C). The structure and purity of the product were confirmed by proton NMR (isopropyl methyl doublet integrating for 6 protons centred at 1·15 ppm), mass spectrometry (MW 376) and elemental analysis (theoretical: C, 57·43, H, 5·36, N, 7·44; found: C, 57·19, H, 5·28, N, 7·28). The melting point of isopropyl-BAPSG was 169–170°C.

Log partition coefficient (log P) values of BAPSG and its prodrugs were calculated using Pallas software (Version 2.0, CompuDrug International, Inc., Budapest, Hungary).

HPLC assay

A reverse-phase HPLC method was used for quantifying BAPSG, methyl-BAPSG, and isopropyl-BAPSG. A Waters HPLC system including a solvent delivery pump (Waters TM 616), a controller (Waters 600 S), an autoinjector (Waters 717 plus), and a PDA detector (Waters 996) were used. The peak areas were integrated using Millennium software (Version 2.15.01). A 25-cm long Microsorb C-18 column (Rainin Instruments, Emeryville, CA) with a particle diameter of 5 μm and a pore size of 100 Å was used. The mobile phase for BAPSG and methyl-BAPSG consisted of acetonitrile, methanol and an aqueous phase (6 mL glacial acetic acid and 7·5 g sodium sulphate per 500 mL water) in a ratio of 20 : 20 : 60 (v/v). The flow rate was maintained at 1·0 mL min^{-1} and the column effluent was monitored at 240 nm for BAPSG and 280 nm for methyl-BAPSG. 2-Nitro BAPSG was used as an internal standard. The retention times for BAPSG, methyl-BAPSG, and 2-nitro BAPSG were 8·0, 17·2, and 6·5 min, respectively. The mobile phase for isopropyl-BAPSG consisted of acetonitrile, methanol and an aqueous phase (8 mL glacial acetic acid and 5 g sodium sulphate per 400 mL water) in a ratio of 40 : 20 : 40 (v/v). The flow rate was maintained at 1·0 mL min^{-1} and the column effluent was monitored at 275 nm. The retention times for isopropyl-BAPSG and BAPSG were 7·0 and 4·5 min, respectively. For all three compounds, the standard graphs ranged from 1·5 to 250 μg mL^{-1} for in-vitro studies and from 0·15 to 20 μg mL^{-1} for in-vivo uptake studies. Samples with concentrations below the lowest limit of standard graphs were designated as N.D. (not detectable).

In-vitro chemical hydrolysis studies

The chemical stability of methyl-BAPSG and isopropyl-BAPSG was assessed in aqueous buffer solutions at pH 1, 5, 6, 7·4 and 9 at 37 ± 0·5°C, and the chemical stability of BAPSG was studied in aqueous buffers at pH 5, 7·4 and 9 at 37 ± 0·5°C. Furthermore, the stability studies for the two prodrugs were conducted at different buffer concentrations (0·025–0·2 M) at pH 7·4 to understand the influence of buffer concentration on the stability. Methyl-BAPSG and isopropyl-BAPSG were expected to hydrolyse to BAPSG in aqueous solutions, with the cleavage of the ester bond being the rate-limiting step.

Reactions were initiated by adding 250 μL of a 10 mM prodrug solution to 4·75 mL buffer in screw-capped test tubes that were pre-equilibrated at $37 \pm 0{\cdot}5^{\circ}$C. BAPSG and methyl-BAPSG stocks were prepared in distilled water. Isopropyl-BAPSG stock was prepared in 50% acetonitrile due to its limited solubility in water. The concentration of acetonitrile in the final reaction mixture was 2·5%. No cloudiness or haziness was observed upon the addition of stocks to appropriate buffer solutions. Unless otherwise stated, all the buffers prepared were of 0·05 M concentration and the ionic strength was maintained at 0·5 for each buffer by adding calculated amounts of potassium chloride. For BAPSG and isopropyl-BAPSG, 200 μL of the sample was collected at predetermined time intervals (0, 6, 12, 18, 24, 36, 48, 72, and 96 h). At the end of each time-point, the reaction was stopped by adding 200 μL acetonitrile–methanol (50 : 50) to the samples. The samples were stored at -20°C until assayed. Exactly 50 μL of each sample was injected onto the HPLC column. For methyl-BAPSG, 200 μL of the sample was taken from reaction vessels and added to 200 μL acetonitrile–methanol (50 : 50) containing 5 μg 2-nitro BAPSG as internal standard. All other steps were similar to those used for BAPSG and isopropyl-BAPSG. Following HPLC analysis, the rate constants for prodrug hydrolysis were determined by linear regression of the logarithm of percentage of the prodrug remaining vs time plots. Triplicate samples were analysed, and the mean rate constant was calculated.

Enzymatic hydrolysis of prodrugs in ocular tissue homogenates

New Zealand White male rabbits were killed by injecting 150 mg kg^{-1} sodium pentobarbital (Sleepaway, Fort Dodge, IA) into the right marginal ear vein. The eyes were enucleated and rinsed with cold saline to remove any traces of blood. Firstly, the aqueous humor was collected using a 23-gauge × 1·5-inch needle attached to a 1-mL tuberculin syringe. A small incision was made in the eyeball in the conjunctival region to aspirate vitreous humor. Subsequently, the cornea, iris-ciliary body, lens, and conjunctiva were separated in a sequence. All tissues were homogenized (Bundgaard et al 1986a), the protein concentration was estimated using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), and the protein concentration was adjusted to 0·5 mg mL^{-1} in all the homogenates. Enzymatic hydrolysis reactions were initiated by adding 200 μL prodrug (10 mM methyl-BAPSG or 5 mM isopropyl-BAPSG) to 1·8 mL tissue homogenate maintained at 37°C and pH 7·4. Exactly 100-μL samples were collected at predetermined time intervals (0, 10, 20, 30, 45, 60, and 90 min) into tubes containing 100 μL acetonitrile–methanol (50 : 50) to inactivate the enzymatic reactions. The tubes were immediately centrifuged at 5000 *g* for 5 min, and the supernatant was collected and stored at -20°C until assayed for the intact prodrug the next day.

Solutions for in-vitro transport studies

Glutathione bicarbonate Ringer's solution (containing in mM: 111·5 NaCl, 4·82 KCl, 0·86 NaH_2PO_4, 29·2 $NaHCO_3$, 1·04 $CaCl_2{\cdot}2H_2O$, 0·74 $MgCl_2{\cdot}6H_2O$, 5 D-glucose, and 0·3 reduced glutathione) was used for the transport studies.

In-vitro transport studies

New Zealand White male rabbits were killed and the rabbit cornea and conjunctiva were isolated and mounted according to the procedure reported by Kompella et al (1992, 1993). After mounting the cornea or conjunctiva in modified Ussing chambers (Navicyte, Sparks, NV), the tissues were exposed to glutathione bicarbonate Ringer's solution containing the drug (BAPSG, 1 mg mL^{-1}; methyl-BAPSG, 450 μg mL^{-1}; isopropyl-BAPSG, 300 μg mL^{-1}) on the mucosal side and neat glutathione bicarbonate Ringer's solution on the serosal side. The fluids were maintained at 37°C and at pH 7·4 under 95% air/5% CO_2 aeration. At predetermined time intervals (0, 30, 60, 120, 180, and 240 min), a 750-μL sample was collected from the serosal side and the lost volume was compensated with neat glutathione bicarbonate Ringer's solution pre-equilibrated at 37°C. The samples collected were centrifuged and 50-μL supernatant samples were directly injected onto the HPLC column for quantifying the prodrug as well as the parent drug. The apparent permeability coefficient (P_{app}, cm s^{-1}) was calculated using the equation, $P_{app} =$ flux/(surface area of membrane × initial concentration), where flux is the slope of the linear portion of a plot of cumulative amount of drug transported vs time.

In-vivo uptake studies

Isotonic solutions at pH 7·1 containing 2 mg mL^{-1} drug, 5% w/v HP-β-CyD (for BAPSG and methyl-BAPSG) or 10% w/v HP-β-CyD (for isopropyl-BAPSG) were used as the dosing solutions. Thirty microlitres of a dosing solution (2 mg mL^{-1}) containing the parent drug or the prodrugs was instilled

directly into the conjunctival cul-de-sac of each eye. At 30 min post-dosing, the rabbit was killed and the corneal and conjunctival surfaces were thoroughly rinsed with normal saline and blotted dry. The ocular tissues including cornea, conjunctiva, sclera, iris-ciliary body, lens, aqueous humor, and vitreous humor were collected in preweighed microtubes, weighed and stored immediately at −70°C. Aqueous humor (50 μL) was directly injected onto the HPLC column. All other tissues were minced, and to the finely ground tissue mass 2 mL acetonitrile–methanol (50 : 50) was added and placed in a shaker for 6 h at 20°C (cold extraction). The samples were then vortexed thoroughly for 5 min and centrifuged at 3000 *g* for 10 min. The supernatant (1·5 mL) was collected in test tubes and the organic layer was dried under N_2 gas (N-EVAP, Organomotion Associates, Berlin, MA). The dried supernatant was reconstituted with 100 μL mobile phase and 50 μL was injected onto the HPLC column. Standard graphs were generated using corneal tissue homogenates every day before the analysis of the samples. The extraction recovery performed at two concentrations (10 and 100 μg mL^{-1}) indicated that the recovery was >95% for the parent drug as well as the prodrugs. Standard curves generated using corneal tissue homogenates were used as a representative of other tissues, which are softer than the cornea.

Mechanisms of BAPSG transport

Corneal permeability of BAPSG (1 mg mL^{-1}) was evaluated in the mucosal to serosal direction in the presence of 100 μM indomethacin (inhibitor of Na^+-monocarboxylate transporter) and 100 μM 2,4-dinitrophenol (inhibitor of oxidative phosphorylation).

Statistical analysis

All values are expressed as mean ± s.d. for n = 3. The statistical significance of differences between the means of the data was evaluated by means of the unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results and Discussion

In-vitro stability

The percentage BAPSG and prodrugs remaining as a function of time at various pH conditions is shown in Figure 2. The rate constants for the degradation of BAPSG and prodrugs in aqueous solutions are shown in Table 1. Of the two esters tested, methyl-BAPSG was less stable when compared with isopropyl-BAPSG at all pH conditions and buffer concentrations. This may be due to greater stabilization of the ester bond with isopropyl substitution compared with methyl substitution. This difference in stability may also be due in part to the differences in their solubility behaviour. Isopropyl-BAPSG was less soluble in aqueous solutions compared with methyl-BAPSG. The greater stability of isopropyl-BAPSG was consistent with previous reports when an increase in chain length made ester prodrugs of 5-iodo-2′-deoxyuridine more resistant to chemical hydrolysis (Narurkar & Mitra 1989). The rate of hydrolysis of methyl-BAPSG was in the order: pH 9>pH 6>pH 7·4/pH 5>pH 1 ($P < 0.05$), and no significant difference was observed in the rate of hydrolysis at different buffer concentrations at pH 7·4. Methyl-BAPSG was highly unstable at pH 9 compared with buffers at low pH ($P < 0.005$), suggesting that the hydrolysis of the methyl ester

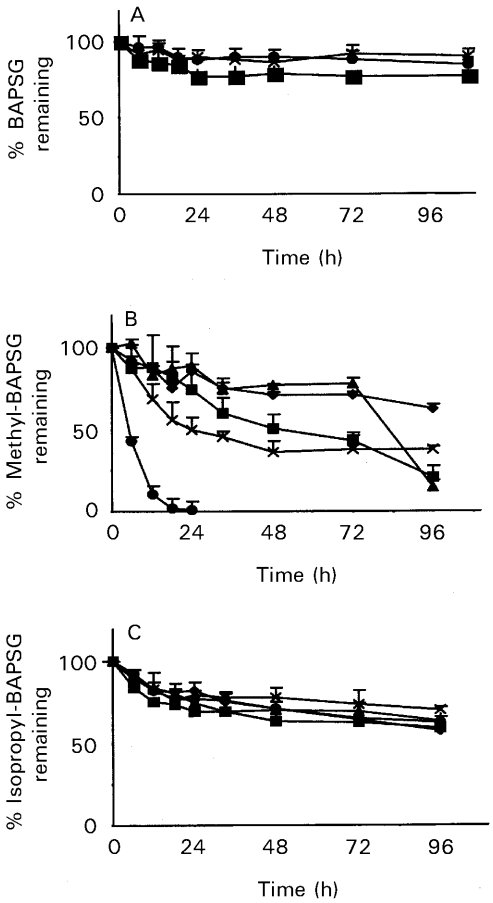

Figure 2. Percent (a) BAPSG, (b) methyl-BAPSG and (c) isopropyl-BAPSG remaining following incubation in aqueous solutions of different pH at 37°C. The ionic strength of all buffers was maintained at 0·5. The data are expressed as mean ± s.d., n = 3. ◆, pH 1·0; ■, pH 5·0; ▲, pH 6·0; ×, pH 7·4; ●, pH 9·0.

Table 1. First-order rate constants for the hydrolysis of 10 mM BAPSG and BAPSG esters at various pH conditions and buffer concentrations.

pH[a]	Buffer concn (M)	Apparent first-order rate constant ($\times 10^{-3} h^{-1}$)		
		BAPSG	Methyl-BAPSG	Isopropyl-BAPSG
1	0·05	N.D.	5·67 ± 1·15	4·45 ± 0·03
5	0·05	8·63 ± 1·3	14·97 ± 3·68	3·72 ± 0·67
6	0·05	N.D.	22·02 ± 4·38	3·35 ± 0·36
7·4	0·025	N.D.	12·44 ± 2·53	3·15 ± 0·14
	0·05	2·51 ± 0·99	11·75 ± 2·53	3·14 ± 1·30
	0·1	N.D.	12·76 ± 1·84	2·01 ± 0·14
	0·2	N.D.	N.D.	2·85 ± 0·41
9	0·05	1·93 ± 0·93	218·9 ± 20·9	4·03 ± 0·14

[a]Ionic strength was maintained at 0·5. Data are expressed as mean ± s.d. for n = 3. N.D., not determined.

was catalysed by alkaline conditions. This can be explained by the general mechanism of hydrolysis of carboxylic esters (Okuyama 1986).

The rate of hydrolysis of isopropyl-BAPSG in aqueous solutions was in the order: pH 1/pH 9 > pH 5/pH 6/pH 7·4 ($P < 0·05$). The stability of isopropyl-BAPSG under acidic and alkaline conditions was probably due to the protection offered by the bulky isopropyl group. At alkaline pH, BAPSG was the most stable of the three compounds. This can be explained in part by assuming the presence of intramolecular hydrogen bonding of BAPSG in aqueous solutions. Possibly for this reason, we observed a change in the UV spectrum of methyl and isopropyl esters of BAPSG compared with BAPSG alone. From the results, it can be observed that methyl and isopropyl group substitution affected the aqueous stability of the prodrug. Thus, ester hydrolysis reduced with increasing chain length of esters. The ester bond appeared to be sensitive at alkaline pH while the parent compound degraded more at acidic pH. Many ophthalmic prodrugs tested previously were O-acyl derivatives that were less stable in aqueous solutions compared with the carboxylic esters tested in this study (Bundgaard et al 1986b; Chien et al 1991).

Enzymatic hydrolysis

Esterases including acetyl cholinesterase and butyryl cholinesterase are abundant in various tissues including ocular tissues (Wistrand et al 1986). Also, ocular tissues possess carbonic anhydrase, an enzyme capable of hydrolysing ester bonds (Wistrand et al 1986; Elleby et al 1999). Consistent with those earlier reports was our observation that ester prodrugs of BAPSG were converted to BAPSG in various ocular tissue homogenates (Figure 3). Although the prodrugs were very stable in aqueous solutions at pH 7·4, they were rapidly hydrolysed to the parent drug in tissue homogenates. This suggests that enzymatic systems must have been responsible for the ester hydrolysis. The prodrugs tested in this study are carboxylic esters similar to PGF2α ester prodrugs tested previously (Camber et al 1986; Camber & Edman 1987).

Methyl and benzyl esters of PGF2α were shown to be hydrolysed by butyrylcholinesterase but not acetylcholinesterase or carbonic anhydrase (Horibe et al 1998). The enzymatic hydrolysis rate constants for methyl-BAPSG estimated in the cornea, conjunctiva, lens, and vitreous humor (Table 2) were not significantly different. The rate of hydrolysis of methyl-BAPSG in iris-ciliary body was less compared with other tissues, contrary to the earlier reports suggesting that hydrolysis of ester prodrugs proceeded most readily in iris-ciliary body (Bundgaard et al 1986b; Narurkar & Mitra 1989). There was no significant difference in the hydrolysis pattern of methyl-BAPSG and isopropyl-BAPSG in tissue homogenates of cornea, lens, aqueous humor, and vitreous humor. The rate of hydrolysis of isopropyl-BAPSG was about

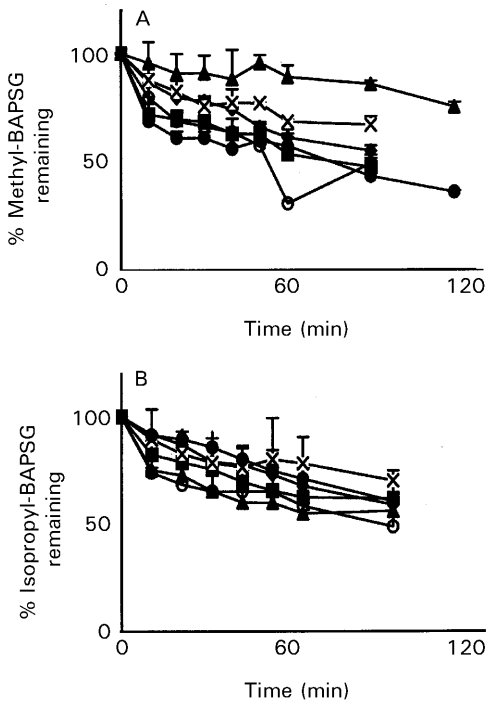

Figure 3. Percent (a) methyl-BAPSG and (b) isopropyl-BAPSG remaining following incubation in various ocular tissue homogenates at pH 7·4 and 37°C. The data are expressed as mean ± s.d., n = 3. ◆, Cornea; ■, conjunctiva; ▲, iris-ciliary body; ○, lens; ×, aqueous humor; ●, vitreous humor.

Table 2. First-order rate constants for the hydrolysis of BAPSG esters in cornea, conjunctiva, iris-ciliary body, lens, aqueous humor and vitreous humor[a].

Tissue	Apparent first-order rate constant ($\times 10^{-3}$ min^{-1})[b]	
	Methyl-BAPSG	Isopropyl-BAPSG
Cornea	6·25 ± 0·69	6·26 ± 2·04
Conjunctiva	7·11 ± 0·57	4·35 ± 0·51
Iris-ciliary body	1·98 ± 0·76	4·01 ± 0·37
Lens	7·20 ± 1·76	6·53 ± 1·31
Aqueous humor	4·13 ± 0·79	3·32 ± 0·88
Vitreous humor	6·85 ± 0·57	5·49 ± 0·14

[a]Experiment was conducted at 37°C and pH 7·4. Protein concentration was adjusted to 0·5 mg mL^{-1}. [b]Data are expressed as mean ± s.d. for n = 3.

2-fold that of methyl-BAPSG in iris-ciliary body homogenates. In conjunctiva, the rate of hydrolysis of methyl-BAPSG was about 1·5-times that of isopropyl-BAPSG. Esterases possess a hydrophobic cavity in their active site (Camber et al 1986), suggesting that the greater the lipophilicity of an ester, the higher is the rate of hydrolysis. However, an increase in the chain length by two units in our study enhanced prodrug hydrolysis in iris-ciliary body only. To understand more about the mechanism of hydrolysis of these compounds, enzyme kinetic studies need to be conducted with specific esterases.

In-vitro transport

The percent of BAPSG, methyl-BAPSG and isopropyl-BAPSG remaining at 37°C in pH 7·4 buffer after 4 h was 99%, 95·4%, and 98·7% (Figure 2), respectively, suggesting that these drugs were relatively stable during the course of in-vitro transport studies. The permeability coefficient of the parent drug (BAPSG) across cornea and conjunctiva was $7{\cdot}44 \pm 1{\cdot}5 \times 10^{-6}$ and $15{\cdot}8 \pm 0{\cdot}27 \times 10^{-6}$ cm s^{-1}, respectively, as reported by Kompella et al (1999). The appearance of methyl-BAPSG and BAPSG during the methyl-BAPSG transport study is shown in Figure 4. Although more lipophilic, the permeability coefficients of methyl-BAPSG were lower (cornea $2{\cdot}17 \pm 1{\cdot}08 \times 10^{-6}$ and conjunctiva $4{\cdot}8 \pm 0{\cdot}9 \times 10^{-6}$ cm s^{-1}). The effective permeability coefficients of methyl-BAPSG, estimated by combining the amounts of BAPSG and methyl-BAPSG in the receiver compartments, were $3{\cdot}92 \pm 1{\cdot}54 \times 10^{-6}$ and $6{\cdot}28 \pm 3{\cdot}06 \times 10^{-6}$ cm s^{-1} for cornea and conjunctiva, respectively. At the end of 4 h, percent methyl-BAPSG transported was less than that of BAPSG (Table 3).

The cumulative amount of BAPSG transported when isopropyl-BAPSG was added to the donor compartment is shown in Figure 5. While the prodrug levels were below detection limits in the receiver chamber at the end of 4 h, BAPSG levels (hydrolytic product of isopropyl-BAPSG) were $4{\cdot}5 \pm 0{\cdot}005$ μg (1·01%) and $21{\cdot}9 \pm 6{\cdot}8$ μg (4·91%) across cornea and conjunctiva, respectively. These levels were lower than those achieved during the BAPSG transport study (Table 3).

The calculated log P values for BAPSG, methyl-BAPSG and isopropyl-BAPSG were 1·09, 1·53, and 2·45, respectively. Since methyl-BAPSG and iso-

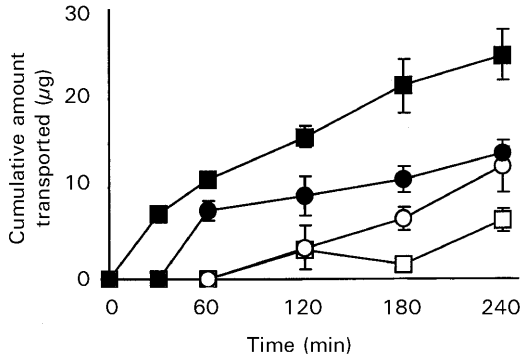

Figure 4. Cumulative amount of methyl-BAPSG and BAPSG transported across rabbit cornea and conjunctiva following mucosal exposure to 450 μg mL^{-1} methyl-BAPSG. The data are expressed as mean ± s.d., n = 3. □, Cornea (methyl-BAPSG); ■, conjunctiva (methyl-BAPSG); ○, cornea (BAPSG); ●, conjunctiva (BAPSG).

Table 3. Effective permeability coefficients and percent transport of BAPSG, methyl-BAPSG, and isopropyl-BAPSG across cornea and conjunctiva[a, b].

	BAPSG[c]		Methyl-BAPSG[d]		Isopropyl-BAPSG[d]	
	Cornea	Conjunctiva	Cornea	Conjunctiva	Cornea	Conjunctiva
P_{app} ($\times 10^{-6}$ cm s^{-1})	7·44 ± 1·49	15·79 ± 0·27	3·92 ± 1·54	6·28 ± 3·06	1·31 ± 0·23	2·7 ± 1·2
% Transport in 4 h	6·27 ± 2·08	14·03 ± 1·71	2·77 ± 0·48	5·7 ± 0·5	1·02 ± 0·09	4·89 ± 1·5

[a]Data are expressed as mean ± s.d. for n = 3. [b]The initial donor concentrations of BAPSG, methyl-BAPSG and isopropyl-BAPSG, were 1 mg mL^{-1}, 450 μg mL^{-1} and 300 μg mL^{-1}, respectively. [c]From Kompella et al (1999). [d]Effective permeability coefficient estimated was based on the total amount of BAPSG and prodrug transported. Effective % transported was estimated similarly.

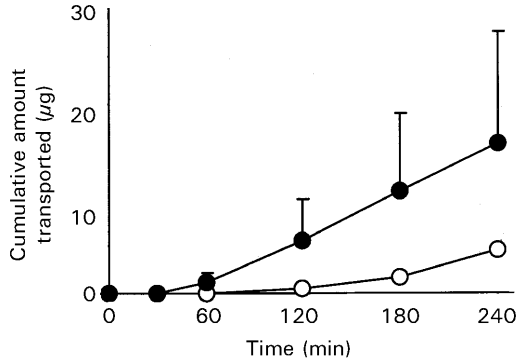

Figure 5. Cumulative amount of BAPSG transported across rabbit cornea and conjunctiva following mucosal exposure to 300 $\mu g\,mL^{-1}$ isopropyl-BAPSG. No isopropyl-BAPSG was detectable on the receiver side. The data are expressed as mean ± s.d., n = 3. ○, cornea; ●, conjunctiva.

propyl-BAPSG are more lipophilic than BAPSG, we expected higher permeability coefficients for these prodrugs similar to the ester prodrugs of timolol and acyclovir (Chang et al 1987; Hughes & Mitra 1993). On the contrary, the permeability was lower for both prodrugs. In our previous studies, lower tissue permeability was observed with phenyl group substitution at the second carbon in the glycine moiety of BAPSG suggesting the importance of unmodified glycine moiety in the transtissue permeability of BAPSG (Kompella et al 1999). The current study suggested that modification of free −COOH could reduce transport of BAPSG.

In-vivo uptake

The ocular tissue levels of the prodrugs and parent drug were estimated using an HPLC assay at 30-min post-dosing (Table 4). Following topical administration of BAPSG, the tissue levels of BAPSG were in the order: conjunctiva > cornea > aqueous humor > iris-ciliary body > sclera > vitreous humor. This seems justified because cornea and conjunctiva were the tissues directly exposed to the formulation and the underlying tissues were aqueous humor and iris-ciliary body. Vitreous humor is a deeper tissue and so lower drug levels were anticipated. With methyl-BAPSG, the intact prodrug levels were higher in the aqueous humor than cornea and conjunctiva. BAPSG was below detection limits following methyl-BAPSG dosing. Following isopropyl-BAPSG dosing, neither the prodrug nor the drug levels were detectable in any of the tissues. The low solubility of isopropyl-BAPSG may be one reason for the low tissue levels.

Table 4. Tissue concentrations of BAPSG, methyl-BAPSG, and isopropyl-BAPSG following single administration at a dose of 60 μg (30 μL drop of a 2 $mg\,mL^{-1}$ solution) to each eye.

Tissue	Tissue concn ($\mu g\,g^{-1}$)[a]		
	BAPSG	Methyl-BAPSG[b]	Isopropyl-BAPSG[b]
Aqueous humor	3·09 ± 0·42	3·29 ± 0·45	N.D.[c]
Vitreous humor	0·01 ± 0·007	0·04 ± 0·03	N.D.
Cornea	5·27 ± 2·13	0·24 ± 0·03	N.D.
Conjunctiva	8·73 ± 3·00	0·31 ± 0·13	N.D.
Iris-ciliary body	2·19 ± 0·72	0·30 ± 0·03	N.D.
Sclera	0·30 ± 0·05	0·25 ± 0·21	N.D.

[a]Data are expressed as mean ± s.d. for n = 3 (three animals, six eyes). [b]No parent drug detectable in ocular tissues following prodrug administration. [c]N.D., not detected (below the lower limit of the standard curve).

With the increase in log P, we expected an increase in uptake of the esters and/or parent drug into deeper ocular tissues, especially aqueous humor. To the contrary, we observed a decrease in the uptake of ester prodrugs compared with parent drug alone (Table 4). This can be explained based on the physicochemical properties of BAPSG. As the pK_a of BAPSG is 3·35 it predominantly exists in its anionic form at physiological conditions. It is possible that such anionic molecules could be transported by carrier-mediated processes such as anion transporters, known to be functional in ocular tissues (Horibe et al 1998; Philip et al 1998). Indeed, we observed that the transport of BAPSG across cornea was reduced from $7{\cdot}44 \pm 1{\cdot}49 \times 10^{-6}$ to $4{\cdot}07 \pm 0{\cdot}7 \times 10^{-6}$ and $3{\cdot}57 \pm 0{\cdot}4 \times 10^{-6}$ $cm\,s^{-1}$ in the presence of indomethacin and dinitrophenol, respectively, suggesting that BAPSG was possibly transported by a Na^+-monocarboxylate transporter in an energy-dependent fashion. Based on this, we speculate that the transport of BAPSG was facilitated by the presence of a carboxylic anion, the lack of which reduced the in-vitro transport and in-vivo uptake of lipophilic prodrugs.

Conclusions

The amount of the drug reaching the intraocular tissues following topical administration of ester prodrugs depends on their tissue permeablility and hydrolysis by tissue esterases. In-vitro stability studies suggested that BAPSG and its prodrugs were relatively stable in physiological buffer conditions. The two esters were hydrolysed in ocular tissues with half-lives ranging from 97 to 350 min. We observed that BAPSG transport and uptake by ocular tissues was reduced by lipophilic ester prodrugs. As the transport of BAPSG was reduced by

an inhibitor of the Na^+-monocarboxylate transporter, and as the modification of −COOH in glycine moiety through prodrug derivatization reduced transport and tissue uptake in this study, it appeared that the −COOH group facilitated the ocular transport of BAPSG.

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