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Research paper

Significance of melanin binding and metabolism in the activity of 5-acetoxyacetylimino-4-methyl-Δ^2-1,3,4,-thiadiazoline-2-sulfonamide[1]

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Abstract

5-Acetoxyacetylimino-4-methyl-Δ^2-1,3,4,-thiadiazoline-2-sulfonamide (compound (1)) is an ester prodrug that lowered intraocular pressure (IOP) in albino New Zealand rabbits, but was found to be inactive in pigmented Dutch Belt rabbits. In order to explain the differences in pharmacological activity for the two rabbit species, metabolism and melanin binding were studied. Depending on the initial concentration, the binding of compound (1) to natural melanin (*Sepia officinalis*) was 20–60%. The binding constant, K, at 37°C was $4.32 \times 10^5\ M^{-1}$ and the maximum moles bound to melanin, r_{max}, was 4.5×10^{-7} mol/mg of melanin. From a determination of binding at temperatures between 25°C and 47°C, a van't Hoff plot was constructed to determine enthalpy and entropy changes accompanying the binding process, ΔH and ΔS, respectively. Values calculated from the plot were −12.7 and −15.4 kcal/(mol deg), respectively. Negative values for these parameters are consistent with charge transfer interactions and therefore suggest that this may be an operative mechanism between compound (1) and melanin. The in vitro incubation of compound (1) was also studied with various ocular tissues from both albino and pigmented rabbits which were iris-ciliary body, intact cornea, stroma/endothelium and aqueous humor. A major metabolite, MET 1, was identified and also observed from in vivo analyses of the same tissues following topical application. The metabolite was isolated and subjected to mass spectroscopy and proton nuclear magnetic resonance spectroscopy analysis. From these analyses, it was hypothesized that the formation of MET 1 involved a GSH conjugation mechanism which displaced the sufonamide ($-SO_2NH_2$) group. The metabolism was found to be less extensive in the pigmented rabbit than in the albino rabbit and suggested that the binding affinity of compound (1) for melanin was a better explanation for the lack of IOP activity in the pigmented rabbit than differences in metabolism.

Keywords: Melanin binding; Metabolism; Intraocular pressure; Carbonic anhydrase inhibitors; Rabbits; Antiglaucoma agents

1. Introduction

Oral carbonic anhydrase inhibitors (CAI) have been used in the treatment of glaucoma for many years. More recently topical application has been employed [1–3]. The most common feature of these drugs is the presence of a free sulfonamide group ($-SO_2NH_2$) linked to an aromatic ring [4,5].

The development of topical CAI's has focused on increasing the potency of a CAI and improving its distribution so as to reach the active site of CA which is intracellular within the ciliary epithelium [6,7]. Whereas improved potency depends on an efficient stereochemical fit with the enzyme, improved distribution to the active site of a topical CAI depends on a number of physicochemical properties of the drug including molecular weight, pKa, partition

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coefficient and solubility. Often during an initial screening process, the lack of activity is attributed to either poor potency or inadequate corneal penetration. However, other more complicating factors may be responsible, such as binding to a non-pharmacological depot or metabolism to an inactive compound. In this study the compound of interest, 5-acetoxyacetylimino-4-methyl-Δ^2-1,3,4,-thiadiazoline-2-sulfonamide (compound (1)), was found to significantly lower intraocular pressure (IOP) in an initial rabbit screening model [8] using New Zealand white rabbits, but when Dutch Belt (pigmented) rabbits were used, no activity was observed. These results prompted us to determine the relative importance of binding to melanin as an explanation for the lack of topical activity of compound (1) and to investigate the potential for metabolism as a contributory factor.

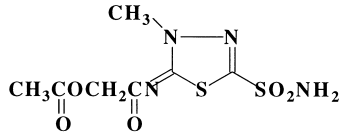
2. Materials and methods

2.1. Materials

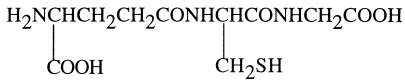
Fig. 1 gives the structure for compound (1). After synthesis and purification, satisfactory proton nuclear magnetic resonance spectra, mass spectra, and carbon/hydrogen/nitrogen elemental analysis (±0.3% of theoretical) were obtained and found to be acceptable. Melting point and percent purity were determined by differential scanning calorimetry (DSC; Perkin Elmer DSC 7 Differential Scanning Calorimeter, Perkin-Elmer, Norwalk, CT, USA). Melting points and purity for compound (1) were found to range from 145.1 to 147.3°C and 98.4 to 99.2% for the different lots that were used in these studies. Methazolamide (MZ; Sigma Chemical, St. Louis, MO, USA), a reference compound for use in comparing results to compound (1), and melanin derived from *Sepia officinalis* (Sigma Chemical) were all used as received.

2.2. High pressure liquid chromatography (HPLC) methodology

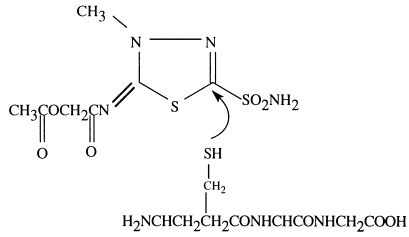
High pressure liquid chromatography (HPLC) was used to assay compound (1) samples. The HPLC system consisted of a solvent delivery system (Model LC-600, Shimadzu, Kyoto, Japan), a variable wavelength UV detector (Model SPD-6A, Shimadzu) and a chart recorder/integrator (Model 601, Shimadzu). Samples were injected on a reverse phase C-18 analytical column (catalog number 112082) μBondapak™ C-18 precolumn cartridge in a Guard-Pak precolumn module (part number 80040 Waters Chromatography Division, Milford, MA, USA). A syringe loading sample injector (Model 7125, Rheodyne, Cotati, CA, USA) fitted with a 100 μl loop (Catalog number 7024, Rheodyne, Cotati, CA, USA) or an automated injector (Model SIL-9A, Shimadzu) was used to inject the samples. The mobile phase was comprised of 90% of 0.1% acetic acid in distilled deionized water and 10% acetonitrile, and



5-imino-4-methyl-Δ^2-1,3,4-thiadiazole-2-sulfonamide HCl



Glutathione



Site of Displacement of Sulfamoyl group by glutathione

Fig. 1. Chemical structures of compound (1) (5-imino-4-methyl-Δ^2-1,3,4-thiadiazole-2-sulfonamide HCl), glutathione (GSH) and the site of displacement of the sulfamoyl group by GSH.

was pumped at a flow rate of 1.5 ml/min. Preliminary results indicated that the intra- and inter-day percent coefficient of variability was <8% for the in vivo samples and <2% for the melanin binding studies.

2.3. IC_{50} potency determinations

Relative in vitro potencies of MZ and compound (1) were measured from the molar concentration of drug which inhibited 50% of the enzymatic activity of carbonic anhydrase II (CA II) that catalyzed the hydration of carbon dioxide (IC_{50}). A pH stat assay was used to determine IC_{50} values according to the method of Maren [9], which has been adapted by other researchers [10] as an in vitro screening tool and used as a procedure in this research for evaluating potency. The pH and the volume of NaOH were recorded as a function of time, and the slope of the linear portion of the curve was expressed as ml NaOH/min. A spontaneous and an enzymatic hydration rate were determined separately. The former contained only the buffer solution in the reaction vessel and the latter contained both the buffer as well as the CA II. A semilog plot of percent inhibition versus log [inhibitor] was used to determine IC_{50}.

2.3.1. Effect of melanin on the IC_{50} of methazolamide (MZ) and compound (1) to CA II

In a separate series of experiments, IC_{50} determinations were made both with and without melanin in order to eval-

uate the effect of melanin on the activity, and hence the binding of compound (1) or MZ to CA II at 4°C. The procedure was conducted as described in the Section 2.3.

2.4. Intraocular pressure (IOP) measurements

The IOP recovery rate assay [8] was used to compare topical activity following administration of compound (1) in New Zealand white rabbits and Dutch Belt pigmented rabbits weighing 5–6 lbs (10–12 weeks old) and Dutch Belt pigmented rabbits weighing 2–3 lbs (10–12 weeks old). Rabbits of approximately equal number of males and females and either species were familiarized with the procedure for 2-3 days prior to making the measurements. An instillation of either a 2% solution (5% hydroxypropylmethyl-cellulose in Sorensen phosphate buffer with 0.05% Tween 80, pH 7.5) or a gel suspension (3% Carbopol 940®, B.F. Goodrich, Cleveland, OH, USA; pH 5.5) was administered to the lower conjunctival sac of a rabbit eye.

The contralateral eye was used as a control and received 50 μl of vehicle only. After 1 h, one drop of 0.5% procaine HCl (Alcaine®, Alcon Laboratories, Fort Worth, TX, USA) was instilled in the eye as a local anesthetic; IOP was measured by applanation tonometry (Model 30D Pneuma-Tonometer, Digilabs, Cambridge, MA, USA). After recording the zero time IOP, a 20% sodium chloride solution was infused into the marginal ear vein using a cassette pump (Scientific Products, McGaw Park, IL, USA) precalibrated to deliver 1 ml/min. After 10 min, the infusion was discontinued and the IOP was again measured every 15 min for 75 min and then every 30 min until the IOP returned to the preinfusion value.

The IOP recovery rate was determined by least squares linear regression for the linear portion of the recovery, which usually represented about eight determinations and yielded an R^2 value of about 0.98-0.99 for 10 measurements made between 45 and 180 min during the IOP recovery period. A minimum of 10 rabbits were used for each formulation and statistical differences between the slopes of the treated and control eyes were tested using a two-tailed *t*-test at a $P = 0.05$ level of significance. The percent decrease in IOP recovery rate was used as a measure of the drug's activity.

2.5. Binding studies with melanin

Different solutions of compound (1) ranging in concentration from 0.05 to 5.5 mM were prepared in 0.1 mM phosphate buffer (pH 7.2). A dry weight of 2.5 mg of *Sepia officinalis* (melanin) was placed in each polypropylene tube (International Equipment, Needham, MA, USA), and a volume of 1 ml of the desired concentration of drug solution was added to each tube. The tubes were equilibrated in a waterbath equipped with a shaker at 37°C for 24 h. Preliminary experiments indicated that compound (1) was stable during the experiment. Melanin, however, was stable through 30 h, but apparently began to degrade since binding results at times over 30 h was reduced considerably with time up to 60 h. After equilibration, the tubes were centrifuged for 15 min in an ultracentrifuge at $30\,000 \times g$ (Beckman Ultracentrifuge, Fullerton, CA, USA). The supernatant was carefully removed and subjected to HPLC analysis to determine the amount of free drug. By knowing the amount of free drug and the total amount initially added, the amount bound was obtained by simple difference. The nonspecific binding of compound (1) to the polypropylene tubes was found to be negligible (<1.2%) at the concentrations that were studied.

The maximum moles of compound (1) bound per milligram of melanin and the affinity constants were calculated from a Langmuir-like adsorption isotherm. It was assumed that the binding was analogous to the adsorption of a drug onto a solid showing monolayer coverage and equivalent binding sites on the melanin surface.

The amount of drug bound per milligram of melanin r, was related to the concentration of free drug, $[D]_{free}$, by Eq. (1):

$$r = \frac{r_{max} K [D]_{free}}{1 + K[D]_{free}} \quad (1)$$

where, r_{max} is the maximum moles bound per milligram of melanin that is the amount needed to form a monolayer, and K is a constant related to the affinity of the interaction.

Rearrangement of Eq. (2) leads to the linear form of the Langmuir adsorption isotherm:

$$\frac{1}{r} = \frac{1}{r_{max}} + \frac{1}{r_{max} K} \frac{1}{[D]_{free}} \quad (2)$$

Using Eq. (2), a linear relation was obtained where $1/r$ was plotted as a function of $1/[D]_{free}$. The extrapolated y-axis intercept from this equation is $1/r_{max}$, and the slope is $1/r_{max} K$. The ratio of the intercept and the slope gave the affinity constant K.

Although the melanin (*Sepia offiinalis*) used in these studies is derived from the ink sac of cuttle fish, chemical evidence [11] indicates that mammalian eumelanins from ox eyes, mouse melanomas, and black human hair are structurally similar. Nevertheless, no reports have been published relating *Sepia offiinalis* to human or rabbit melanin located in iris tissue.

2.5.1. Additional binding studies with melanin

Similar experiments were conducted in duplicate and averaged at 25°C, 30°C, 37°C and 47°C. A van't Hoff plot was constructed to determine the thermodynamic parameters for the binding process in order to gain insight into the type of binding between compound (1) and melanin. As a comparison of binding affinity for compound (1), MZ binding to melanin at 37°C was also determined at a constant concentration of 0.9 mM of compound (1) and a range of concentrations of MZ from 0.25 to 3.5 mM at 37°C. In a similar set of experiments, the potential displacement of

compound (1) (0.9 mM) by MZ (0.25-3.5 mM) from melanin (2.5 mg) was studied at 37°C.

2.6. Metabolism studies

2.6.1. Collection of tissues and preparation of homogenates for in vitro analysis

New Zealand white rabbits of either sex, weighing 5-6 lbs (10–12 weeks old) were sacrificed with 1/2 ml of Beuthanasia-D Special® (pentobarbital sodium 390 μg/ml and phenytoin sodium 50 mg/ml; Schering, Kenilworth, NJ, USA). The latter preparation was mixed with 1/4 ml of normal saline which was administered by intravenous injection into the marginal ear vein. The same procedure was used to sacrifice Dutch Belt rabbits of either sex weighing 3-4 lbs (13–14 weeks old). Aqueous humor was aspirated by inserting a 26 gauge needle at the corneal scleral junction. The syringe was adjusted to contain 20 μl of air. The eye was proptosed from its socket and the needle inserted anterior to the limbus, above the iris, at an upward angle into the anterior chamber. The needle was inserted slightly upward in order to reduce the possibility of sampling vitreous humor. The air was discharged from the syringe into the anterior chamber and aqueous humor was withdrawn. The air bubble and increased pressure in the anterior chamber allowed for easier visualization and removal of the aqueous humor. The aqueous humor was transferred to a 2 ml polypropylene tube, capped and placed on dry ice.

After rinsing the eye with normal saline and gently blotting away excess fluid, cornea samples were obtained by first making a small incision in the tissue with a 9 mm trephine (Storz Instrument, St. Louis, MO, USA) and then completing the removal with the use of a pair of corneal scissors (Storz Instrument). The tissue was rinsed a second time with normal saline and blotted to remove residual aqueous humor. For intact cornea, the epithelial cells were scraped gently from the surface of the eye using a scalpel blade (Storz Instrument). These tissues were also stored in dry ice in 2 ml polypropylene tubes. Tissues from both the eyes of the rabbits were pooled together to obtain enough tissue homogenate.

Iris-ciliary body samples were removed from the ocular cavity with forceps (Storz Instrument) by gently grasping the iris and detaching it as a single piece of tissue. It was rinsed with normal saline, blotted to remove excess fluid and stored in polypropylene tubes on dry ice.

All of the ocular tissue samples were weighed. To the cornea and iris-ciliary body samples, eight volumes of ice-cold 0.25 M ultra pure grade sucrose (Boehringer Mannheim, Indianapolis, IN, USA) with 1 mM Dithiothreitol (DTT; Boehringer Mannheim) was added and the samples were homogenized at 500 rev./min for 2 min using a Teflon® pestle connected to a motor driven power unit (Con-Torque®, Eberbach, Ann Arbor, MI, USA). DTT is a protective agent for sulfhydryl groups. Aqueous humor samples were diluted with eight volumes of ice-cold 0.25 M sucrose with 1 mM DTT. The tissue homogenates were kept at 4°C in an ice bath until they were incubated with drug solution.

2.6.2. Incubation of tissue homogenates with compound (1)

A 0.5 mM solution of compound (1) was prepared in 0.1 mM phosphate buffer (pH 7.2) with 1 mM DTT. A volume of 0.7 ml of a solution of compound (1) was added to a Duall® homogenizer tube (Kontes Glass, Vineland, NJ, USA) containing 0.5 ml of tissue homogenate. The mixtures were incubated in a 37°C water bath for 60 min. A control mixture containing buffer without drug, and a control mixture containing sucrose solution with DTT without tissue homogenate were identically treated. The mixtures were extracted and analyzed for drug.

2.6.3. Extraction of drug from tissues

Cold 0.01 N hydrochloric acid (0.5 ml) was added to the iris-ciliary body and cornea samples in the Duall® tissue homogenizer. After 15 min at 4°C, the samples were homogenized at 500 rev./min for 2 min using a fritted glass pestle turned by a motor driven power unit. An additional 0.5 ml aliquot of 0.01 N hydrochloric acid was added to each tube. The contents of the tube were mixed for 1 min. The aqueous humor samples were not homogenized. A volume of 1 ml of 0.01 N hydrochloric acid was added and mixed with the aqueous humor samples. These tissue samples were extracted with three 2 ml aliquots of ethyl acetate (EtOAc) by adding EtOAc to glass tubes secured with a Teflon® lined screw cap followed by 5 min of mixing. The tubes were centrifuged (Model centra-7R, International Equipment, Needham Heights, MA, USA) at 3500 rev./min for 15 min at 4°C to separate the aqueous and EtOAc layers. The upper EtOAc layers from each of the three extractions were transferred to a 10 ml disposable culture tube (Fisher Scientific, Pittsburgh, PA, USA) and evaporated to dryness under a gentle stream of nitrogen. Once dried, the tubes were capped and stored at −20°C until reconstituted with mobile phase for HPLC analysis.

2.6.4. In vivo studies to confirm the presence of metabolites

This experiment was performed to confirm the presence of the metabolites in vivo. New Zealand white rabbits of either sex, weighing 5-6 lbs (10-12 weeks old), were anesthetized with an intramuscular injection of a mixture of ketamine (Aveco, Fort Dodge, IA, USA; 31.6 mg/kg), acepromazine (Aveco; 0.75 mg/kg), and xylazine (Mobay, Shawnee, KA, USA; 5.1 mg/kg). The drugs were injected in the right thigh 15 min before the start of the experiment using a 25 gauge × 5/8 inch needle attached to a 5 ml disposable syringe and providing anesthesia to the rabbits for 50–80 min. A second injection of the anesthetic mixture was often necessary after 60 min.

The rabbits were placed on their left sides with their bodies secured in large plastic bags. The eyelids of the anesthetized rabbits were kept open by an eye speculum

(Storz). A polymethylmethacrylate cylinder [12], the base of which was designed to fit the curvature of the eye, was secured along the corneal scleral junction of the right eye by a cyanoacrylate adhesive (Nexaband®, CRX Medical, Raleigh, NC, USA). The left eye of each rabbit remained undisturbed.

A volume of 300 μl of a 44 μg/ml solution of drug in 0.07 M phosphate buffer (pH 7.65) was placed inside the cylinder. The solution in the cylinder was replaced with fresh drug solution every 15 min to maintain a constant drug concentration exposed to the corneal surface. At the end of 2 h of infusion, drug solution in the cylinder was aspirated and the cylinder was removed. The eye was rinsed with 0.9% sodium chloride and gently blotted dry. The rabbit was sacrificed with a 1 ml intravenous injection of 75% (v/v) Beuthanasia-D Special® in normal saline into the marginal ear vein.

Aqueous humor, cornea and iris-ciliary body tissues were removed and treated as described previously. All tissue samples were temporarily stored in dry ice until they could be stored in a −20°C freezer. Drug was later extracted from thawed samples and analysis of the extracted drug was performed by HPLC.

2.6.5. Identification of metabolite (MET 1)

In vitro incubation experiments (60 min), which included the iris-ciliary body, were designed in order to provide sufficient metabolite (MET 1) for structural analysis. The concentration of compound (1) was increased 2-fold to 1 mM and in another experiment the number of eyes were increased in order to increase the amount of enzyme available in the tissue homogenates. A control incubation experiment was conducted without the iris-ciliary body to observe changes, if any, in the MET 1 peak.

A solution of 0.5 mM of compound (1) was prepared in 0.1 mM phosphate buffer along with 1 mM DTT adjusted to a pH of 7.2 with ammonium hydroxide and kept for a week at 37°C. A volume of 500 μl of this solution was injected on a reverse phase C-18 column. The MET 1 peak was collected from the column at a retention time of 25 min. Approximately 10 fractions were collected, pooled, frozen at −20°C and then lyophilized. The lyophilized sample was analyzed using an HPLC system equipped with a photodiode array detector (Model SPD-M6A, Shimadzu, Kyoto, Japan). The lyophilized samples were then subjected to structure determination techniques which were proton nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry.

2.6.6. Proton nuclear magnetic resonance spectroscopy

Proton NMR spectral analyses were performed using either a 6000 or a 10 000 Hz spectral width (NMR Facility, Director Dr. W.R. Kearney, College of Medicine, The University of Iowa, IA, USA; Varian UNITY-500 MHz spectrometer). The larger spectral width was used to ensure that sulfonamide resonance positions were not folded in from larger chemical shifts. Spectra were collected using 90 deg pulses, an acquisition time of 5 s and a relaxation delay of at least 8 s. Transitions were averaged until the signal-to-noise ratio was adequate for accurate integration of the most dilute species of interest. The sample solutions were prepared in deuterated dimethyl sulfoxide (DMSO; Isotec, OH, USA) containing 99.96 atom% D.

2.6.7. Mass spectrometry

The samples were analyzed by ionization techniques, partly by fast atom bombardment (FAB-MS) and partly by electrospray (ESI-MS). The equipment consisted of a VG Analytical ZAB-HF reversed geometry using a BE configuration, where B is a magnetic sector and E is an electrostatic analyzer, equipped with an Ion Tech, saddle-field FAB gun and commercial FAB ion source. Samples were bombarded with 8 X keV Xe atoms with an atom gun current of 1.5 mA. The FAB matrices that were used were 3-nitrobenzyl alcohol, glycerol and thioglycerol. Samples were dissolved in water followed by the addition of 1–2 μl of the solution to the matrix on the FAB probe tip.

All ES ionization mass spectra were collected using a prototype VG ZAB-T four-sector mass spectrometer. Full-scan spectra were obtained by scanning the MS1 with a resolving power of 100 over the mass range of 100–1000 at a rate of 10 s/decade (a 1 s delay between the scans). For ES, the accelerating potential was 4 kV and the collision cell was floated to 2 kV for MS/MS experiments. A solution of CsI and a mixture of PEG 400 and 600 were used to calibrate the mass spectrometer during full voltage scan experiments, respectively.

The spray needle of the VG ES source was maintained at 8000 V, the counter electrode at 5000 V, and the sampling cone, skimmer lens, skimmer, hexapole and ring electrode were typically maintained at 4177, 4125, 4117, and 4116 V, respectively. Nitrogen was used as a bath and nebulizer gas with a flow rate of approximately 300 and 12 l/h, respectively. The bath gas temperature was 80°C and a solution of 90:10 water/acetonitrile was infused to the spray needle at a rate of 10 ml/min. Samples were flow injected (Rheodyne 7125 injector, Cotati, CA, USA). A 20 μl loop was used for full voltage scan experiments.

2.6.8. Reaction of compound (1) with glutathione (GSH)

This experiment was carried out to investigate the role of glutathione (GSH, reduced form; Sigma Chemical) in the formation of the metabolite (MET 1). A solution of 0.5 mM compound (1) in 0.1 mM phosphate buffer (pH 7.2) and 1 mM GSH was incubated at 37°C for 4 h. The solution was then injected onto a reverse phase C-18 column periodically to observe changes in the initial drug solution. Control experiments were performed by incubating the phosphate buffer (pH 7.2) with 1 mM GSH at 37°C for an hour in comparison to a phosphate buffer solution (pH 7.2) with 1 mM GSH without incubation.

3. Results

3.1. IC_{50} potency determinations

The molar concentration of the inhibitor which would inhibit 50% of the enzymatic activity of CA II in catalyzing the hydration of carbon dioxide (IC_{50}) was determined for compound (1) and MZ. These values are 124.3 and 142.1 nM, respectively. Although comparisons of IC_{50} values to determine relative potencies cannot confidently be made between different laboratories because of different enzyme activities and different experimental conditions, large differences, i.e., 5–10-fold or more, likely indicate a greater potency for the smaller IC_{50} value. Consequently, dorzolamide (Trusopt®, Merck) with a reported IC_{50} of 0.2 nM [3], would be considered much more potent than compound (1) or MZ. The very high potency attributed to dorzolamide is a significant factor in explaining its ability to lower IOP when applied topically to the eye. Much higher doses of MZ must be given which is only effective by the oral route of administration. In fact, the relatively similar IC_{50} value obtained for compound (1) in comparison to MZ would likewise predict inactivity for compound (1) when administered topically to the eye.

3.2. Intraocular pressure (IOP) measurements

The recovery rate slopes of treated and control New Zealand white rabbits and Dutch Belt pigmented rabbits, along with statistical analyses, are listed in Table 1. Interestingly, it was found that compound (1) was topically active ($P < 0.05$) in the New Zealand white rabbit, but not in the pigmented rabbits ($P > 0.05$), when compared to the respective control vehicles. In ophthalmic drug development, the IOP recovery rate assay is often used as an initial screening tool prior to testing the drug for its ability to lower IOP in a glaucomatous monkey eye [3]. Although the New Zealand white rabbit is most often used for initial screening, the pigmented Dutch Belt rabbit is an important species for further testing before testing in either monkey or human eyes because of differences in each species relative to melanin binding or to metabolism [13–18]. Clearly, for compound (1), the large difference in results for the two rabbit species suggests that metabolism and/or binding to melanin are primary factors contributing to the difference in activity.

Table 1

Summary of intraocular pressure (IOP) recovery rates for compound (1) in two vehicles after a single dose of 50 μl to eyes of albino New Zealand and Dutch Belt rabbits

Formulation	% decrease in IOP activity[a]	Number of eyes
New Zealand white rabbits		
2% solution of compound (1) in HPMC[b]	34.3*	10
2% suspension of compound (1) in carbomer gel[c]	37.2*	10
Dutch Belt rabbits		
2% solution of compound (1) in HPMC[b]	5.85**	8

[a]Comparison of treated vs. vehicle control recovery slopes using IOP recovery rate model [8].
[b]5% hydroxypropyl methylcellulose (Methocel®) in Sorensen phosphate buffer with 0.5% Tween 80, pH 7.5.
[c]3% Carbopol® gel, pH 5.5.
*$P < 0.05$, **$P > 0.05$.

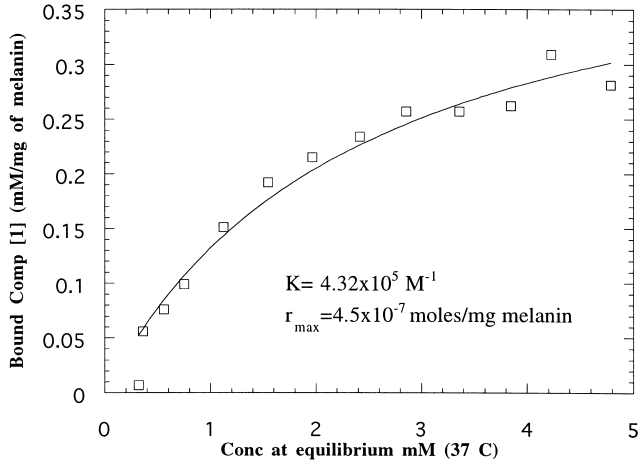


Fig. 2. Langmuir-like plot for the binding of compound (1) to melanin (*Sepia officinalis*) at 37°C.

3.3. Binding studies with melanin

In preliminary experiments, the effect of incubation time of compound (1) with melanin (*Sepia officinalis*) showed that a plateau was reached between 12 and 24 h. Therefore all of the studies were performed with an incubation time of 24 h. Fig. 2 shows a typical isotherm of bound compound (1) in mM/mg of melanin when plotted against free drug concentration at equilibrium (37°C). The shape of the curve indicated that binding to melanin followed a Langmuir-like adsorption isotherm, which was consistent when the binding of other drugs with melanin was studied [14–21]. Binding ranged from 20 to 60% depending on the initial concentration of compound (1). Using non-linear least squares regression, the average ($n = 4$) binding constant, K, at 37°C, was $4.32 \pm 0.96 \times 10^5\ M^{-1}$ and the r_{max} was $4.57 \pm 0.58 \times 10^{-7}$ mol bound/mg of melanin (Table 2).

3.3.1. Effect of temperature on the binding of compound (1) to melanin

Fig. 3 was used to determine the thermodynamic parameters for the binding of compound (1) to melanin at 25°C, 30°C, 37°C and 47°C. The values of the enthalpy and entropy, ΔH and ΔS, that were calculated from a van't Hoff plot were −12.7 kcal/mol and −15.4 cal/(mol deg), respectively. Negative values for these parameters are consistent with charge transfer interactions [22] and therefore suggest a participation of charge transfer between com-

Table 2

Summary of binding parameters for the interaction of carbonic anhydrase inhibitors and melanin at different temperatures

	Maximum moles bound per milligram of melanin[a] (r_{max})	Affinity constant (K) M^{-1}
Methazolamide		
37°C	$1.97 \pm 0.27 \times 10^{-7}$	$6.16 \pm 1.29 \times 10^{5}$
Compound (1)[b]		
25°C	$1.37 \pm 0.31 \times 10^{-7}$	$8.04 \pm 3.90 \times 10^{5}$
30°C	$2.07 \pm 0.21 \times 10^{-7}$	$6.97 \pm 2.19 \times 10^{5}$
37°C	$4.57 \pm 0.58 \times 10^{-7}$	$4.32 \pm 0.96 \times 10^{5}$
47°C	$1.62 \pm 0.64 \times 10^{-6}$	$1.92 \pm 0.46 \times 10^{5}$

[a]*Sepia officinalis*.
[b]5-Acetoxyacetylimino-4-methyl-Δ^2-1,3,4,-thiadiazoline-2-sulfonamide.

pound (1) and melanin. In general, a charge transfer interaction is most likely to occur between large aromatic molecules or cyclic structures with electron donating substituents, such as oxygen, nitrogen and sulfur moieties [23]. Based on the thermodynamic results obtained, other forces may also be involved in the binding, in particular, hydrogen bonding and the weaker the Van der Waals forces.

Melanin, which possesses a free radical, forms an irreversible charge transfer interaction in which the melanin becomes an electron acceptor and the ligand (e.g., compound (1)) becomes the electron donor. Melanin (*Sepia officinalis*) is a polyanion, whose structure is not completely understood, but is known to undergo reversible redox reactions and contains carboxyl and phenolic groups [11,24]. The sulphonamide moiety of compound (1), along with its heterocyclic ring structure that provides sufficient lipophilicity, is likely responsible for the binding to melanin [13]. The negative values of enthalpy and entropy are consistent with charge transfer interactions [22] and as the binding between the donor and acceptor become stronger, ΔH can be expected to have a large negative value. The specificity of interacting sites (e.g., structural restraints) also become greater as the strong charge transfer interactions increase, leading to a large negative ΔS value [22].

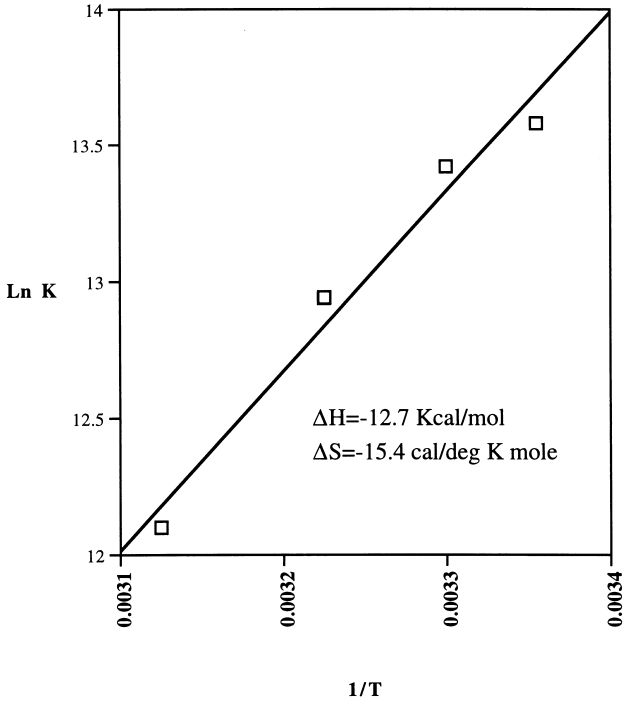


Fig. 3. van't Hoff plot for the binding of compound (1) to melanin (*Sepia officinalis*) at 25°C, 30°C, 37°C and 47°C.

3.3.2. Binding of MZ and the competitive inhibition of compound (1) to melanin

Table 2 gives the values for K and r_{max} that were calculated from non-linear least squares regression of a plot of bound MZ or compound (1) in mM/mg of melanin vs. free drug at equilibrium (37°C). For MZ, the values of K and r_{max} are $6.16 \pm 1.29 \times 10^5$ M^{-1} and $1.97 \pm 0.27 \times 10^{-7}$ mol/mg of melanin, respectively. The affinity constant of MZ is of the same magnitude but about 50% greater than that of compound (1), indicating that MZ has about the same affinity for melanin, thus requiring a similar concentration of MZ to effectively compete with compound (1) for binding to melanin. The concentration of MZ required to inhibit 50% of bound compound (1) to melanin (IC_{50}) was 3.14 mM.

3.3.3. Effect of melanin on the IC_{50} of methazolamide (MZ) and compound (1) to CA II

This experiment was conducted to determine the extent to which melanin would compete with CA II for the binding of MZ or compound (1). In the eye, melanin and CA II are anatomically and kinetically separated by rate-determining barriers, so that in vivo, melanin would not directly compete with CA II for a CAI. However, significant binding to melanin could prevent enough drug from reaching CA II so that a lowering of IOP would be affected.

This experiment was designed to determine the molar concentration of compound (1) that would inhibit 50% of the enzymatic activity of CA II in catalyzing the hydration of carbonic anhydrase in the presence and absence of melanin (IC_{50}). Table 3 lists the IC_{50} values of compound (1) and MZ in the presence and absence of melanin at 4°C. The experiment could not be conducted at 37°C because the spontaneous hydration of CO_2 was too rapid at this tempera-

Table 3

IC_{50} Values of methazolamide and compound (1)[a] determined for the inhibition of carbonic anhydrase II with and without melanin

Compound	IC_{50} (nM)
Compound (1)	112.0, 124.2[b]
Compound (1) in presence of melanin[c]	120.6
Methazolamide	142.1
Methazolamide in presence of melanin	151.2

[a]5-Acetoxyacetylimino-4-methyl-Δ^2-1,3,4,-thiadiazoline-2-sulfonamide.
[b]Determined with a separate lot of carbonic anhydrase; other values were determined from the same lot.
[c]*Sepia officinalis*.

ture for an accurate measurement. At 4°C the results indicate that no significant difference exists between the IC_{50} values and can be explained on the basis that CAI's are known to have high affinities for CA II, on the order of $>10^9$ M^{-1} [9] as compared to 10^5 M^{-1} for the binding of compound (1) to melanin.

3.4. Metabolism studies

Although the binding of compound (1) to melanin could explain the difference in IOP activity between white and pigmented rabbits, conversion to an inactive metabolite is also a possibility. From the structure of compound (1), it was theorized that lack of activity in the pigmented rabbit could result from ester hydrolysis, which is more prominent in the pigmented species. This reaction could occur specifically on the *O*-acetyl side chain attached to the imine nitrogen to produce 5-imino-4-methyl-Δ^2-1,3,4-thiadiazoline-2-sulfonamide HCl. This latter compound has been shown to have no IOP lowering capability when dosed topically (3% suspension) to the eyes of white rabbits, male for female [25].

3.4.1. Detection of metabolites following incubation of compound (1) with ocular tissues

Following incubation of compound (1) for 1 h at 37°C with intact cornea, stroma/endothelium, aqueous humor or iris-ciliary body in phosphate buffer containing 1 mM DTT, two metabolites (MET 1 and MET 2) and the drug peak were detected at retention times of 25, 16 and 34 min, respectively (see Fig. 4). Chromatograms representing incubation samples that included ocular tissue but not compound (1) were used as controls. When compound (1) was incubated without tissue, the presence of MET 2 was detected at equivalent peak areas of the HPLC chromatogram, indicating that its formation is a result of a degradation process. At 4°C, a temperature at which enzymatic activity is considerably reduced, lower peak areas for MET 1 were detected. Fig. 5 shows the results from 4°C and 37°C for the incubation of each ocular tissue. At 37°C HPLC peak areas of MET 1 formed from compound (1) were the greatest in iris-ciliary body, followed by stroma/endothelium and intact cornea. Very little MET 1 was detected in aqueous humor. In preliminary experiments, the maximum rate of formation of MET 1 increases to 1 h at which time it maintains a plateau up to 3 h, the maximum time period the incubations were conducted. At 4°C, no MET 1 was detected in aqueous humor, but significant amounts were detected in the iris-ciliary body, followed by stroma/endothelium and intact cornea. The relative amounts of MET 1 formed in the different ocular tissues varies, but corroborates very well with the levels of enzymes present in these ocular tissues [26–30]. Results showed no difference for male and female rabbit eyes which also agrees with previous findings [26].

At 60 min prior to incubation at 37°C, the ocular tissues

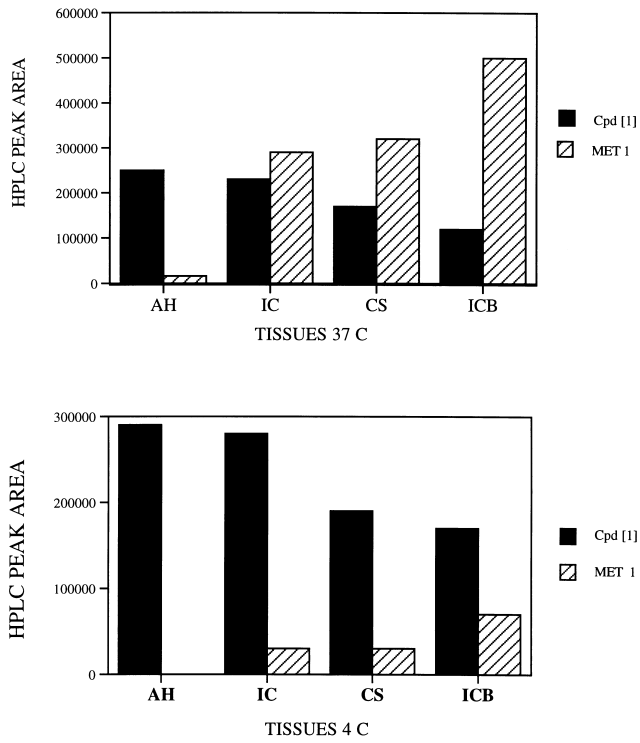


Fig. 4. HPLC peak height areas representing the amount of compound (1) and major metabolite (MET 1) formed upon in vitro incubation (60 min) at 4°C and 37°C for aqueous humor (AH), intact cornea (IC), stroma/endothelium (CS) and iris-ciliary body (ICB).

were heated for 10 min at 60°C to further validate the enzymatic hydrolysis of compound (1). The chromatograms of these incubations showed no formation of MET 1. However, the formation of MET 2 was unaffected.

The incubation experiments were also conducted in ocular tissues excised from Dutch Belt rabbits. Their ocular tissues, when incubated with compound (1), showed a much reduced formation of MET 1 and only through the first 20 min of incubation.

3.4.2. Detection of metabolites following in vivo instillation of compound (1)

Following the maintenance of a 0.0044% solution of compound (1) for 1 h on the cornea of anesthetized rabbits, ocular tissues (intact cornea, aqueous humor and iris-ciliary body) were removed, and then extracted for analysis of drug and metabolite. Chromatograms for all three tissues showed the formation of MET 1, whereas, the MET 2 peak was not apparent.

3.4.3. Identification of MET 2

Evidence indicated that MET 2 was not an ocular metabolite; nevertheless, it was of interest to attempt to identify its structure. The large number of rabbit eyes necessary to collect sufficient MET 2 prevented NMR and mass spectroscopy analyses. Although the latter techniques are essential to structural identification, various compounds were injected onto the HPLC column in order to provide a clue

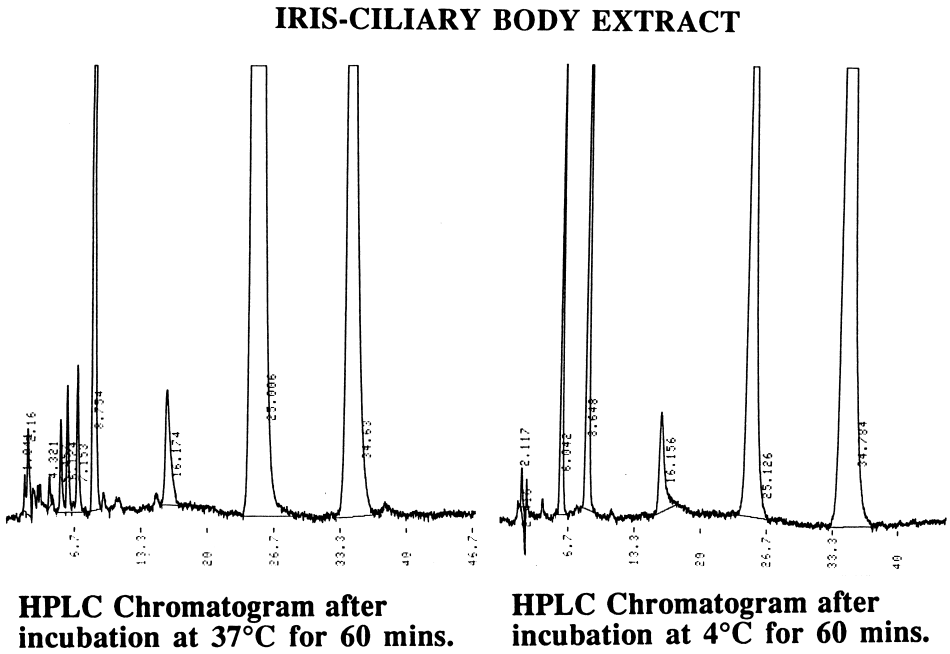


Fig. 5. HPLC chromatogram of iris-ciliary body extracted from New Zealand white rabbits showing a minor metabolite (MET 2), a major metabolite (MET 1) and compound (1) at retention times of 16, 25 and 35 min, respectively.

as to the structure of MET 2. The compound, 5-imino-4-methyl-Δ^2-1,3,4-thiadiazoline-2-sulfonamide HCl, was a starting material for compound (1) and was injected onto the HPLC column. *p*-Toluene sulfonic acid was also injected onto the column assuming a $-SO_3H$ compound was produced as opposed to the $-SO_2NH_2$ from the original compound (1). Because of the wide differences in retention times for these two compounds compared to compound (1), their structures were rejected from consideration. However, when MZ was injected onto the column, the exact retention time of 13 min was observed. The mobile phase was changed to methanol/water (20:80) and again an identical retention time for the two compounds was observed but at 11.6 min because of the change in mobile phases. When both MET 2 (obtained by collecting the MET 2 fraction from an incubation sample) and MZ were mixed together in phosphate buffer (pH 7.2), a single peak at a retention time of 11.6 min was again measured. Further analyses using a photodiode array UV-VIS detector (Shimadzu, model SPD-M6A, Kyoto, Japan) suggested a single peak for the mixture. Although MZ provides a lead for the identification of MET 2, a metabolic mechanism for its formation is not clear.

3.4.4. Identification of MET 1

Preliminary experiments with mass spectroscopy indicated that sodium and acetate ions in the mobile phase interfered with the analysis. Therefore, a mobile phase of 90:10 acetonitrile/water was used in the collection of MET 1. For FAB-MS three separate matrix systems, 3-nitro benzyl alcohol, glycerol and thioglycerol, were used. The results in each of the three matrix systems indicated the presence of a base ion at m/z 158. Other ions, which were much less significant, were identified at m/z 174, 214, 256 and 414. ESI-MS was used to confirm the presence of the molecular ions identified by FAB-MS and is shown in Fig. 6. For this procedure, the base peak was observed at 158.1, with lesser peaks observed at m/z 174.1, 214.2 and 256.2. Other ions appeared at m/z 351.3, 413.3, 483.2, 649.0 and 806.2, with the latter ion appearing to be a cluster of ions representing m/z 158 and 649.

High resolution mass spectrometry (HRESI) was performed on the two most abundant ions at m/z 158.1 and 413.3. HRESI results indicated that the exact isotopic composition of the two ions was 158.1828 and 413.3509.

MS-MS experiments, using an electrospray ionization source and subjecting the ions to collisional activation, were also performed to determine the fragmentation patterns of the most abundant ions (see Figs. 6 and 7). The ion at m/z 806 produced ions at 649 and 158. The ion at m/z 413 produced fragmentation ions at 256 and 158, whereas the ion at m/z 351 produced only one fragment at 158. The ion at m/z 158.1 was the most abundant ion and was present in all of the other fragment patterns, indicating that it is the most stable ion and that each of the ions contains the basic molecular structure that accounts for the molecular weight of 157 (i.e., $M^+ + H = 158$). The base peak at m/z 158 produced a fragmentation pattern that began with m/z 142 (a loss of 16) followed by m/z 128, 114, 100, 84, 71, 58 and 44 which mostly represent repetitive differences of m/z 14 (see Fig. 7).

[^{1}H]NMR analyses of MET 1 showed a spectrum indicat-

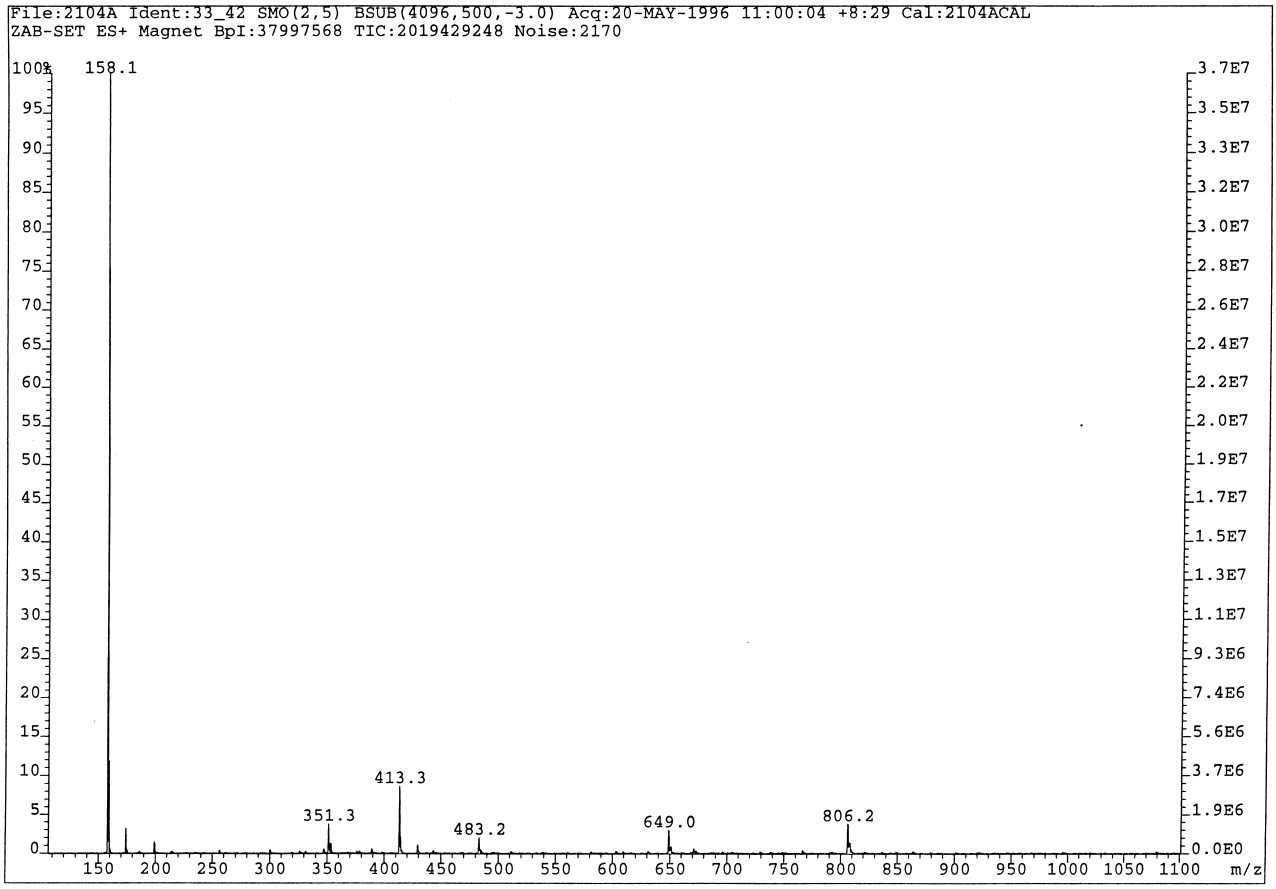


Fig. 6. Fragmentation pattern of electron spray mass spectroscopy (ESI-MS) of the major metabolite (MET 1) extracted from the incubation of compound (1) and iris-ciliary body tissue.

ing the absence of the -NH_2 group and the presence of a -N-CH_3 group on the thiadiazole ring of compound (1). These results indicated that the conjugated ring system of compound (1) is part of the structure of MET 1 and that its formation could be occurring via a GSH conjugation mechanism.

3.4.5. Reaction of compound (1) with glutathione (GSH)

The structure of GSH is included in Fig. 1, which also shows the site of displacement of the sufamoyl group by GSH. Incubated samples of a solution of 0.5 mM compound (1) in 0.1 mM phosphate buffer (pH 7.2) with 1 mM GSH showed a peak at a retention time midway between the retention times of compound (1) and MET 1. After incubation of compound (1) and GSH, 7 and 35% of compound (1) was converted to the unknown midpoint peak at the end of 3 and 4 h, respectively. Although reactions with GSH are usually catalyzed by GSH-*S*-transferases, a number of compounds have been reported to undergo non-enzymatic conjugation with GSH at alkaline pH, but at a slower rate of formation than in the presence of an enzyme [31]. In 1990, the conjugation of MZ and GSH was identified by Khishida et al. [31], which suggests that the formation of MET 1 is also a result of a GSH conjugation mechanism.

4. Discussion

The unequal pharmacological activity of compound (1) in the albino and pigmented rabbits led to binding studies of compound (1) and melanin as well as to studies examining the differences in the extent of metabolism in the two rabbit species. The binding studies of compound (1) to natural melanin (*Sepia officinalis*) indicated binding to the extent of 20–60%, depending on the initial concentration of compound (1). Significant binding of a CAI to melanin would allow for a non-pharmacological depot within the eye and in relatively close physical proximity to the active site. Although the large difference in affinities of CAI's for CA II compared to melanin ($>10^9$ M^{-1} vs. $>10^5$ for compound (1) to melanin) favors a shift of the equilibrium to CA II, this shift in equilibrium may be less dramatic in vivo. Within the eye, the location of CA II (intracellular epithelium of the ciliary process) is anatomically separated from the melanin (iris) by a number of barriers. These barriers also significantly reduce the ability of drug to shift rapidly from melanin to CA II. Also, if the capacity of melanin for compound (1) is relatively large compared to the capacity of CA II for compound (1), then the melanin site could provide a sink which would prevent drug from reaching CA II very

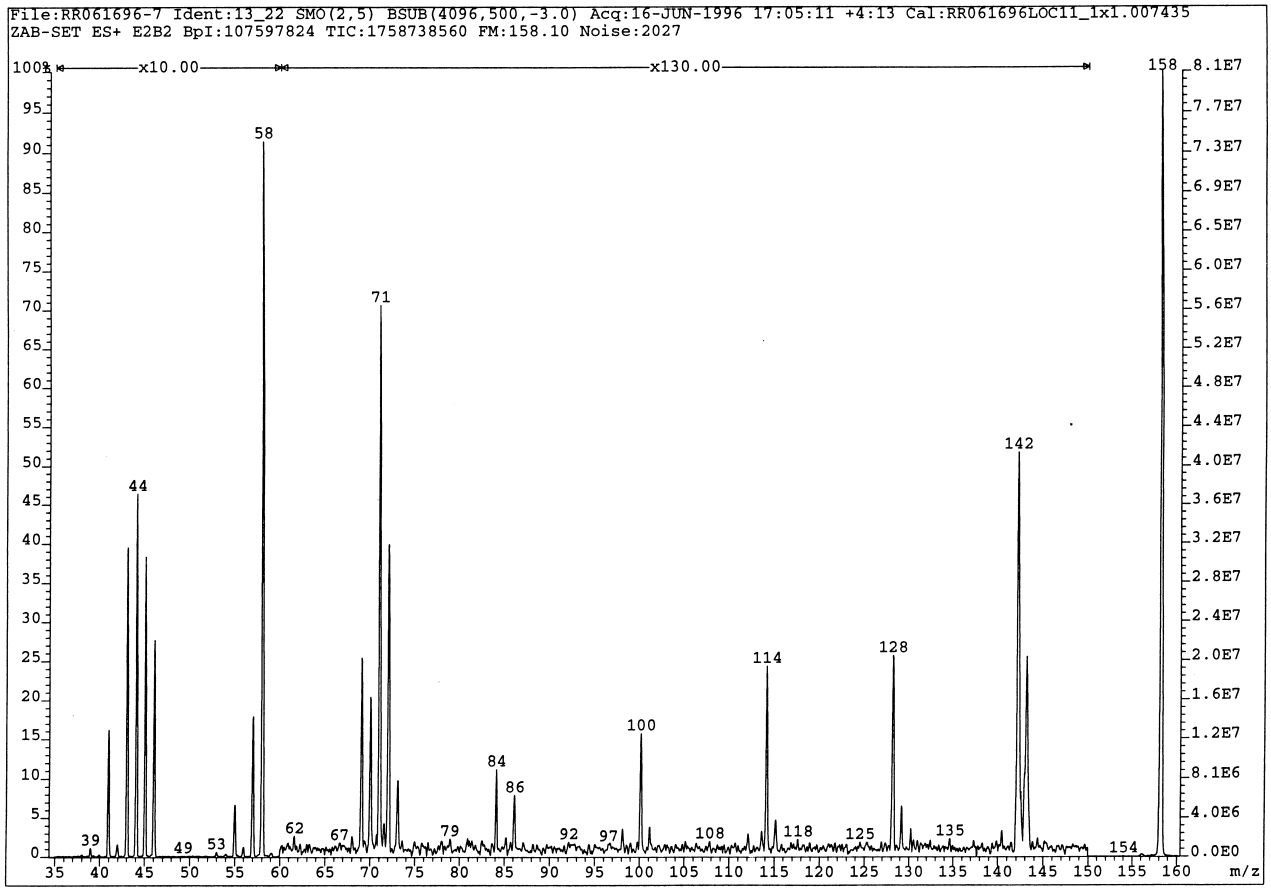
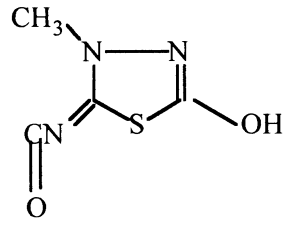


Fig. 7. Fragmentation pattern of MS-MS spectroscopy of the base ion m/z 158 obtained from the fragmentation of the major metabolite (MET 1) extracted from the incubation of compound (1) and iris-ciliary body tissue.

rapidly. This is particularly important with regard to the inhibition of CA II to lower IOP because of the large turnover of CA II and the fact that over 99% of CA II must be bound before a lowering of IOP can occur [9]. Therefore, any non-pharmacological depot that is capable of accumulating a significant amount of CAI, could reduce the drug's activity to lower IOP. If the binding capacity of the melanin within the iris was sufficient, the phenomenon would significantly prevent drug from rapidly reaching the active site. The higher concentration of melanin located within the iris of the pigmented rabbit eye compared to the albino rabbit eye could explain the lack of IOP in the pigmented rabbits.

A single major metabolite (MET 1) of compound (1) was identified in iris-ciliary tissue and, to some extent, in other ocular tissues according to a rank order of iris-ciliary body > stroma/endothelium > intact cornea > aqueous humor. The extent of metabolism was greater in albino rabbits than pigmented rabbits, which is contrary to what was expected based on the knowledge that pigmented rabbits have higher levels of esterase enzymes [32]. From the MS and [^{1}H]NMR chromatograms, it was deduced that: (1) the m/z 158 fragment is the most abundant ion, (2) the thiadiazole ring is a basic chemical entity, and (3) GSH likely results in a displacement of the (-SO_2NH_2). The following structure was proposed as a fragment ion that provides a lead to the identification of the structure of MET 1:



Further likelihood that GSH is involved in the formation of MET 1 was reasoned from the fact that reactions with GSH are catalyzed in vivo by GSH-*S*-transferase [33]. Both GSH and GSH-*S*-transferase are present in various tissues of the rabbit eye, the iris-ciliary body exhibiting the highest GSH-*S*-transferase activity [34]. However, the ratio of reduced (GSH) to oxidized (GSSH) GSH is determined by the presence of GSH reductase [35], the latter of which depends on the presence of melanin pigmentation [35,36]. Based on this information, it is possible that pigmented rabbits have lower levels of GSH and GSH reductase resulting in lower metabolism and lower formation of MET 1 compared to albino rabbits.

Therefore, assuming that the metabolite is less active in

lowering IOP because of a loss of the $-SO_2NH_2$ moiety, the greater metabolic activity in albino rabbits would favor a reduced IOP in this rabbit species. Since greater IOP activity was observed in the white rabbits, the affinity of compound (1) for melanin better explains the pharmacological differences in IOP activity (i.e., albino rabbits lowered IOP to a much greater extent than pigmented rabbits). These findings stress the importance of using pigmented, as well as non-pigmented rabbit eyes, in screening new drugs for glaucoma since rabbits are often used prior to a final evaluation in glaucomatous monkey eyes in identifying a clinical candidate.

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