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Research Paper

Pharmacokinetics and Disposition of Memantine in the Arterially Perfused Bovine Eye

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Purpose. To develop an improved ([1]This is to clearly acknowledge that we have tried to improve an existing model.) arterially perfused bovine eye model and investigate the general ocular disposition of memantine.
Materials and Methods. Fresh bovine eyes were prepared by exposing and cannulating one ciliary artery, placing the eye into a perfusion chamber and slowly increasing the rate of perfusion to 1.0 ml/min. Analysis of the arterial perfusion pressure (APP), intraocular pressure (IOP), venous perfusate for glucose consumption and lactate dehydrogenase (LDH) activity, and histopathology ensured viability. Memantine was administered with the perfusate (simulated systemic access), by an intravitreal injection and by topical infusion. At the appropriate time points, the cornea, aqueous humour, sclera, iris-ciliary body, choroid/RPE, retina and vitreous humour were harvested and analysed for memantine.
Results. The preparation remained viable for at least 9 h. At this time, histopathological examination showed mild to moderate deterioration of retinal layers. However, all retinal layers remained well defined and the integrity of the inner limiting membrane and Bruch's membrane were preserved. Glucose consumption, LDH levels and constant APP and IOP showed that correct cannulation and viability was maintained. After administration, memantine accumulated in the melanin rich iris-ciliary body and choroid/RPE. Results following topical administration indicate that substantial concentrations of memantine are present in the retina and choroid/RPE.
Conclusions. The arterial perfused bovine eye system proved to be a useful system for ocular drug delivery studies. The experimental results indicate that memantine will accumulate in the posterior segment when delivered by the topical route and that melanin-binding may support sustaining significant concentrations in the retina.

KEY WORDS: arterial perfused bovine eye; melanin binding; memantine; ocular drug delivery; retinal drug delivery.

INTRODUCTION

The Perfused Bovine Eye

The arterially perfused mammalian eye preparation is a versatile model for ophthalmic research and has been used for the measurement of pharmacological (1–4), metabolic (5–7), drug delivery and pharmacokinetic (8) parameters in ocular studies. A perfused organ offers several advantages over *in vivo* experiments involving sedated and anaesthetised animals. For example, isolation of the eye from the central nervous, hormonal and vascular systems avoids extra-organ regulatory effects which might occur in the intact animal as a reflex during anaesthesia, and thus complicate the interpretation of experimental results (3,9). In addition, such a preparation permits complete control over the circulatory supply with respect to nutrients and physiological factors (10). Drugs may be administered to the preparation at any concentration (9) and models of disease states such as experimental uveitis may be induced while avoiding animal suffering (11).

Research groups described arterially perfusion preparations for multiple species, including feline (12,13), bovine (14), canine (15), monkey (1), frog (16), rabbit (9), rat (17), guinea pig (4) and equine eyes (11). Despite the increasing variety of species, preparations of feline and bovine eyes remain the most widely used.

In the bovine eye, the central retinal artery provides blood flow to the inner retinal layer with the two long ciliary arteries being responsible for supply of nutrients to all other ocular structures (18). These arteries merge within the eye

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ABBREVIATIONS: AAP, arterial perfusion pressure; EDTA, ethylenediamine-tetra acetic acid; GCL, ganglion cell layer; H&E, hematoxylin and eosin; INL, inner nuclear layer; IOP, intraocular pressure; LDH, lactate dehydrogenase; ONL, outer nuclear layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium; S-MEM, serum-free minimal essential medium.

and it has been shown that cannulation and perfusion of one of the ciliary arteries is sufficient to maintain the eye's viability for an extended period (3,8,9,20). The primary vessels of the venous system are the vortex veins which exit the globe near the equator (18).

Perfusion of the eye may be performed either by techniques of constant pressure or of constant flow. Benefits of perfusion at constant pressure are the reduced risk of damage to the blood-retinal and blood-aqueous barrier; however, compared to constant flow perfusion the intraocular pressure (IOP) may show greater variability (3). The flow rate of the perfusate may also vary considerably with the potential risk of insufficient nourishment of the ocular tissues, especially if the experiment involves the administration of vasoconstrictive compounds. Perfusion at constant flow provides the eye with a continuous supply of nutrients, but at high flow rates the risk of vascular leakage (11) and retinal oedema or haemorrhage (19) may increase. Concerns such as these necessitate the monitoring of parameters that permit the observation of pathophysiological changes in order to determine the validity of the experiment.

To ensure the structural integrity of ocular tissues and maintenance of viability, a number of parameters may be used to assess the validity of a particular perfusion experiment. Depending on the intended objectives of the study these include: electroretinogram, IOP and arterial perfusion pressure (APP) or flow, glucose consumption, lactate dehydrogenase (LDH) activity, oxygen consumption and carbon dioxide generation (3,11,19–24). Histopathological and immunohistochemical examinations of ocular structures to assess morphological changes occurring during perfusion can be performed (9,20,22).

One objective of these studies was to develop an isolated perfused bovine eye system which would be viable for prolonged periods. Although in previous studies our laboratory maintained the viability of perfused bovine eye preparations for a maximum period of 4 h (8) an extended period is necessary for studying the ocular disposition and metabolism of drugs. Furthermore, a system which maintained enzyme systems at a vital level may yield insight into the manner which a delivered drug altered the metabolic pathways of the eye.

Ocular Disposition of Memantine

An additional objective of this study was to examine the general disposition of memantine (Fig. 1) in the perfused bovine eye following different routes of administration. Achieving therapeutic concentrations in the posterior segments of the eye from topical ocular administration is thought to be impossible. However, previous *in vivo* studies have shown that memantine is a potential candidate for topical delivery to the posterior segment of the eye.

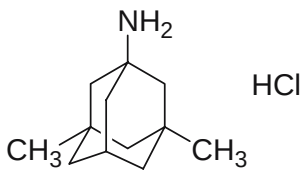

Fig. 1. Structure of memantine hydrochloride. Molecular formula $C_{12}H_{21}N{\cdot}HCl$; molecular weight 215.76 (free base 179.20); partition coefficient, log(*P*) 3.28; basicity, pK_a 10.42; solubility of the hydrochloride salt 3.5% in a pH 6.5 aqueous solution at 25°C.

Memantine hydrochloride (1-amino-3, 5-dimethyladamantane hydrochloride, Fig. 1) has neuroprotective properties mediated by blocking the calcium channels activated by *N*-methyl-*D*-aspartate (NMDA) receptor stimulation (24–26). Excessive activation of NMDA receptors is thought to be responsible for the calcium-dependent neurotoxicity associated with neurodegenerative diseases. Currently, memantine is used for the treatment of Alzheimer's disease. In addition, its neuroprotective properties suggest that it may be of benefit for the treatment of glaucoma. The mode of action is thought to be due to the prevention of damage to retinal ganglion cells which results from the increased intraocular pressure (27,28).

In a study investigating the melanin-binding characteristics of memantine it was demonstrated that melanin has a high affinity and capacity to bind memantine (29). Although these results were obtained under *in vitro* conditions, it was thought likely that memantine would accumulate in melanotic tissues (e.g., iris-ciliary body and RPE) despite the presence of important physiological barriers (cell membranes).

The physico-chemical characteristics of memantine (Fig. 1) would suggest that memantine diffuses significantly across the lipophilic corneal epithelium and hydrophilic corneal stroma (30,31). While the distribution coefficient is the rate limiting factor for corneal transport, scleral permeability is thought to be determined primarily by molecular weight and size (32,33). Thus the amphiphilic properties and low molecular weight of memantine would potentially make it a candidate which would permeate the cornea and sclera. The high lipophilicity of memantine should facilitate its diffusion across the outer blood-retinal barrier, the RPE. It is thought that melanin-binding could create a posterior depot of memantine facilitating delivery across the RPE into the posterior of the eye.

The perfused bovine eye preparation was used for the investigation of the ocular disposition of memantine since it provides control over many physiological parameters while viability and structural integrity are maintained.

MATERIALS AND METHODS

Chemicals and Reagents

Serum-free Eagle's minimum essential medium for tissue culture (product number M4767), sodium bicarbonate, ethylenediamine-tetra acetic acid (EDTA, free acid), penicillin G sodium salt, streptomycin, gentamycin free base, insulin from bovine pancreas, bovine holo-transferrin (siderophilin) iron saturated, atropine sulfate, sodium selenite and phosphate buffered saline tablets (product number P4417) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Glucose test kit based on Sigma procedure 635, LDH test kits based on Sigma procedure 500 and DG1340-UV, enzyme controls (normal, 2-N) and Accutrol normal chemistry control serum were also purchased from Sigma-Aldrich. Sodium hydroxide pellets and hydrochloric acid (1.0 M) were purchased from Mallinckrodt-Baker (Phillipsburg, NJ, USA). A medical grade gas mixture of 95% oxygen and 5%

carbon dioxide was obtained from Praxair (Danbury, CT, USA).

Acetonitrile (HPLC grade) and hydrochloric acid (1.0 M) were purchased from Mallinckrodt-Baker. Hexane and methanol were of HPLC grade and obtained from Burdick & Jackson (Muskegon, MI, USA). Memantine hydrochloride, boric acid and sodium borate decahydrate were provided by Allergan. Formic acid and 2-adamantanamine hydrochloride were purchased from Sigma-Aldrich.

Instrumentation

Bovine eyes were perfused using a peristaltic cassette pump (Monostat, New York, NY, USA). Perfusion medium and eyes were maintained at constant temperature using a RMSG6 circulating waterbath (MGW Lauda, Lauda, Germany). All glassware including condensers (used as heat exchangers) and custom made perfusion chambers were purchased from ACE Glass (Louisville, KY, USA). The pressure recording system consisted of TranStar disposable transducers and connecting cables (Medex, Dublin, OH, USA) and signal conditioners CM015 10 V bridge, adapters CM002 ADC16, data logger ADC16 high resolution data logger and data logging software Picolog (Picotech, St. Neots, UK).

Absorbance measurements for the determination of LDH activity and glucose concentration were performed using a Beckman DU-70 UV/VIS spectrophotometer (Beckman-Coulter, Fullerton, CA, USA). Buffer solution pH values were measured with a Beckman-Coulter Φ10 pH meter, calibrated prior to use with Beckman-Coulter pH standard buffer solutions.

LC-MS analyses were performed with a HP1100 series HPLC system connected to a HP1100 MSD mass spectrometer, both from Agilent Technologies (Palo Alto, CA, USA). Data acquisition and integration was controlled by Agilent Technologies ChemStation software. Tissue extractions were achieved using a rotational shaker (Labquake, Kansas City, MO, USA), an Accuspin centrifuge from Beckman-Coulter and a tissue tearor, model 985, 370 Type 2 (Biospec Products, Bartlesville, OK, USA).

Perfusion System

The perfusion of bovine eyes with perfusate saturated with a gas mixture of 95% oxygen and 5% carbon dioxide was at a constant flow of 1.0 ml/min (Fig. 2). The perfusion apparatus setup maintained the eyes at 37°C and recorded the arterial perfusion and intraocular pressure at regular intervals. Parts *e* to *m* of the system (Fig. 2) were present in triplicate, allowing the perfusion of three eyes in parallel.

A gas mixture of oxygen (95%) and carbon dioxide (5%) was continuously bubbled in the perfusion medium while being stirred. This was performed for at least 45 min prior to perfusion to ensure that the medium was saturated. A peristaltic pump with Tygon tubing (1.6 mm I.D., 3.2 mm O.D., Formulation R-3603, Saint-Gobain Performance Plastics, Akron, OH, USA) was used to deliver perfusion medium to the bovine eye. A filter (Nylon Acrodisc, 0.2 μm pore size, 25 mm diameter, Gelman Sciences, Ann Arbour, MI, USA) was used to prevent particulates entering the eye and to reduce the amount of potential biological contaminants. The perfusion medium was warmed up to physiological temperatures by directing it through the coil of a condenser (200 mm jacket length). The outlet of the condenser was connected to a bubble trap (9.5 mm I.D., 15.9 mm O.D., silicone tubing, Baxter, Deerfield, IL, USA), a 3-way-stopcock (nylon, Kontes, Vineland, NJ, USA) and a pressure transducer to record the arterial perfusion pressure.

The custom made perfusion chamber resembled a jacket-heated beaker with an outlet in the bottom (Fig. 2). The internal diameter and depth of the chamber were 80 and 120 mm, respectively. To increase humidity in the perfusion chamber, while allowing drainage of the venous perfusate, a funnel (70 mm diameter, polyethylene, VWR, San Diego, CA, USA) was inserted into the bottom opening and sealed.

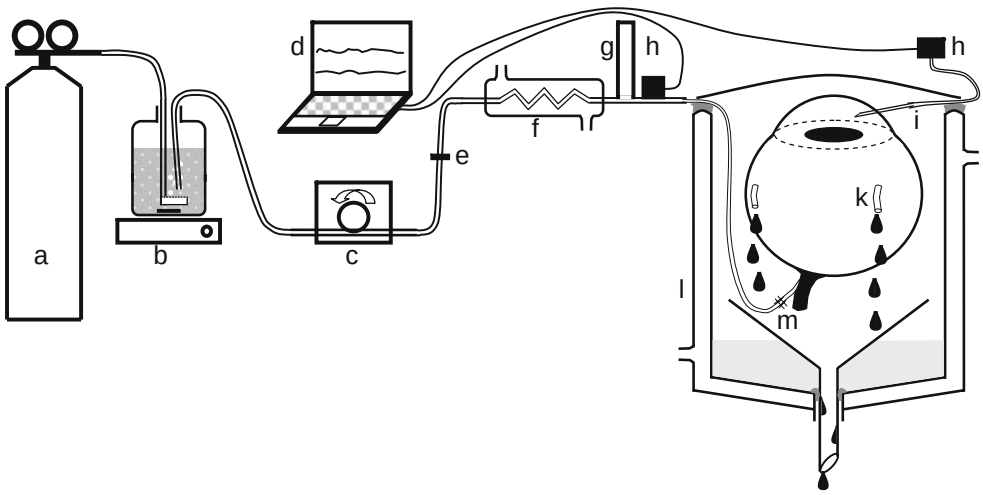

Fig. 2. Setup. Schematic diagram illustrating the perfusion system. (a) Gas mixture of 95% oxygen and 5% carbon dioxide; (b) magnetic stirrer and perfusate bubbled with gas mixture; (c) peristaltic pump; (d) computer system recording pressure readings; (e) filter; (f) heat exchanger; (g) bubble trap; (h) pressure transducer measuring APP; (i) needle and cannula measuring IOP; (k) vortex vein; (l) jacket-heated beaker; (m) cannulated ciliary artery.

Approximately 40 ml of deionised water were placed in the space surrounding the funnel. A net consisting of four wires attached to three quarters of a ring (~12 mm diameter) was used to suspend the eye in the perfusion chamber while avoiding obstruction of the optic nerve and ocular blood vessels. Modelling clay was placed on the rim of the chamber to seal it with a watch glass while allowing entry of the cannula providing perfusion medium and measuring the IOP. All male and female luer connectors were barbed and had an I.D. of 1.59 mm (Upchurch Scientific, Oak Harbour, WA, USA).

Preparation of Bovine Eyes

Bovine eyes obtained from Sierra Medical Sciences (Santa Fe Springs, CA, USA) were received in a plastic bag on ice within approximately 2 h of slaughter. Immediately upon receipt the eyes were removed from the ice and allowed to warm up to ambient temperatures. Excessive adnexal tissue was carefully trimmed off to avoid damage to the superficial blood vessels of the posterior globe. An approximately 10 mm wide part of the conjunctiva, adjacent to the limbus, was left attached.

For cannulation, one of the long ciliary arteries was exposed and cleared. A tapered piece of polyethylene tubing attached to a luer connector was inserted in the artery and secured with a suture. Prior to cannulation the cannula was filled with perfusion fluid to avoid the inclusion of air bubbles and thus risk of formation of emboli. In order to prevent the cornea, or other tissues, from drying out, the eye was kept moist with perfusion medium during the preparation procedure.

Short transport times between slaughterhouse and laboratory followed by rapid perfusion are vital to achieve adequate viability during the complete course of perfusion. It is beneficial if the eyes are delivered with a large amount of adnexial tissue as this protects the eyes, arteries and veins during transport. Though rapid delivery from slaughterhouse to laboratory was established the exact age of the cows could not be obtained.

Perfusion Procedure

For a baseline pressure-measurement the recording of the arterial perfusion and intraocular pressure was started shortly before the perfusion of the eye. The cannulated eye was placed in the wire net of the jacket-heated beaker with the cornea facing upwards. The cannula was then attached to the perfusion system whilst taking care to avoid the presence of air bubbles in the perfusion line. Perfusion was commenced at a flow rate of about 0.4 ml/min and increased to 1.0 ml/min within 45 min. Immediately after starting perfusion the vortex veins were inspected for flow and if necessary cut to initiate venous flow.

To record the IOP the pressure transducer, the line (silicone tubing, 0.76 mm I.D., 1.65 mm O.D., Baxter) and needle (23 G, Beckton Dickinson, Franklin Lakes, NJ, USA) were filled with PBS free of air bubbles and the needle inserted into the anterior chamber. Perfusate was used to rinse any tissue particles and blood off the globe and to moisten the eye. In order to reduce evaporation and to prevent the eye from drying out a small piece of polyethylene film (cling-film) was placed loosely on the cornea. Care was taken to avoid disturbing the IOP measuring needle, and the assembly was sealed with a watch glass on top of the jacket-heated beaker affixed with modelling clay.

Perfusion Medium

The perfusion medium used was based on studies by de Coo *et al.* (20), where it was shown that viability of bovine eyes could be sustained for a maximum period of 9 days by supplementing a serum-free minimal essential medium (S-MEM with Earle's salts and L-glutamine) with some essential serum substitutes and antibiotics. While antibiotics minimise the risk of bacterial and fungal contamination accessory factors such as insulin, transferrin and sodium selenite are vital to normal cell metabolism and thus extend the period of viability. The pH of the perfusion medium was adjusted to 7.4 with sodium hydroxide (1.0 M) immediately after it was saturated with a gas mixture of 95% oxygen and 5% carbon dioxide for a minimum period of 30 min.

Viability Parameters

Arterial Perfusion Pressure (APP)

The pressure transducer for the measurement of APP was connected via a three-way-stopcock which was placed between a bubble trap and the perfusion chamber (Fig. 2) and filled with perfusion medium free of air bubbles. Pressure was monitored every 5 s over the entire period of perfusion.

Intraocular Pressure (IOP)

Measurement of the IOP was facilitated by a needle in the anterior chamber connected to a pressure transducer. The needle was inserted 5–10 min after perfusion commenced. The pressure transducer was aligned with the centre of the eye to avoid siphoning effects. Prior to needle insertion the assembly was filled with PBS free of air bubbles. The recording rate was as described for APP.

Glucose Consumption

The measurement of glucose in the perfusion medium and venous perfusate was based on the Sigma procedure 635 (Sigma-Aldrich), with some modifications. The test kit contained a glucose standard solution (10.0 mg/ml) and *o*-toluidine reagent.

Calibration solutions were prepared by mixing fresh perfusion medium (900 μl) with various volumes of glucose standard solution and deionised water, giving a final volume of 1,000 μl.

For the measurement of glucose, aliquots (40 μl) of the calibration solution and collected venous perfusate were transferred separately to glass vials (4 ml, VWR) and *o*-toluidine reagent (2.0 ml) was added. Samples for the measurement and subtraction of background absorbance were prepared by mixing deionised water (40 μl) with *o*-toluidine reagent (2.0 ml). The closed vials were placed in a boiling waterbath for exactly 10 min, followed by quick

cooling to room temperature. The solution was then transferred to disposable semi-microcuvettes (1.5 ml, VWR) and absorbance was measured at 635 nm within 45 min of preparation.

For the quantification of glucose the standard addition method was used. In this procedure the absorbance of the calibration solutions was plotted against the added glucose concentration. Linear regression was used to determine the slope of the curve. The glucose concentration of the samples was calculated as the quotient of the absorbance of the sample to the calculated slope with a factor of 0.9 to account for dilution.

Lactate Dehydrogenase (LDH) Activity

The activity of LDH in samples of venous perfusate was determined using one of two methods. This was necessary as the test kit which was used initially (Sigma procedure 500) was withdrawn by the supplier, requiring the use of an alternative (Sigma procedure DG1340-UV).

These test kits were used according to the manufacturers instructions. Test kit performance was ensured by using enzyme controls (Sigma procedure 500) and Accutrol normal chemistry control serum (Sigma procedure DG1340-UV) according to the manufacturers instructions.

Analysis and Quantification of Tissue Samples

The conditions for the analysis of the ocular tissues by LC-MS were as described by Koeberle *et al.* (34) except that the mass spectrometer was operated in scanning mode, collecting mass spectral data in the range 100 to 300 m/z. Briefly, memantine was chromatographed on a reversed-phase column (Prodigy 5 μm, ODS (3), 100 Å, 100 × 4.6 mm) using gradient elution with mobile phases of 0.1% formic acid in deionised water and 0.1% formic acid in methanol at a flow rate of 0.8 ml/min.

From these mass spectral data single ion traces at mass-to-charge ratios (m/z) of 152 (2-adamantanamine) and 180 (memantine) were extracted and the analyte peaks integrated for quantification.

Minitab 13 (State College, PA, USA) was used to assess calibration curves by linear regression of peak area ratios of memantine to 2-admantanamine *versus* memantine concentration; a weighting factor of $1/x^2$ (where x is the concentration of memantine HCl) was used. In the analytical sequence samples were regularly bracketed by sets of four standard solutions.

All tissue samples (cornea, aqueous humour, sclera, vitreous humour, retina, choroid/RPE and ICB) were prepared for analysis by a liquid–liquid extraction procedure. 2-Admantanamine, which is structurally similar to memantine, was used as internal standard (IS) to account for variability of the extraction process.

In the process of extraction the sample was made alkaline to convert analyte and IS completely into their lipophilic, non-ionised forms which were then extracted with hexane. To improve efficiency of the extraction, the tissue samples had to be extracted three times with hexane. Acetonitrile was added to some tissue samples to break an emulsion layer that formed during extraction which could not be broken by centrifugation. For the extraction of the cornea and sclera it was necessary to extract memantine and IS into an aqueous phase first from which, after alkalisation, they were extracted with hexane. This step was necessary as the direct addition of hexane to the tissues caused the formation of an emulsion layer that could not be broken by an addition of acetonitrile. Furthermore, the extraction of the analytes into the aqueous phase was supported by acidifying the samples which converts the analytes quantitatively into their ionised forms, hence increases their hydrophilicity. The dry residues were reconstituted in HPLC mobile phase and analysed in duplicate.

Validation of Extraction and LCMS Method

The extraction procedure and LCMS method used for the analysis was validated for selectivity, precision, accuracy and linearity. Validation was performed for every ocular tissue that was analysed (cornea, aqueous humour, sclera, vitreous humour, ICB, choroid/RPE and retina).

Selectivity was shown by single peaks for 2-adamantanamine (t_R = 1.9 min) and memantine (t_R = 3.6 min) which were not present in the respective single ion chromatograms of blank samples prepared from either perfused eyes (3, 4.5 and 9 h) or freshly enucleated bovine eyes.

Precision of recovery after extraction with respect to retention time and peak area ratio of memantine to IS was determined by the replicate extraction of ocular tissues spiked with aqueous memantine hydrochloride solution. The different ocular tissues were spiked by adding known quantities of memantine hydrochloride to the individual tissue and shaking for 18 h. Results for the precision of the extraction process (recovery) for the various ocular tissues showed that the retention times of memantine and IS varied less than 5% and peak area ratio of memantine to IS by less than 17%.

Accuracy values were determined from the analysis of ocular tissues spiked with memantine. The results showed that accuracy varied between 45 and 167% of the nominal concentration. To account for these great differences in accuracy, the obtained values were used to calculate the correct concentration of memantine in tissue samples.

Results from weighted linear regression and test for zero-intercept (34) of four sets of standard solutions prepared in HPLC mobile phase (A/B 50:50 v/v) showed that variability of slope and intercept were within acceptable limits. Furthermore, correlation coefficients indicated linearity ($r^2 \geq 0.99$). For all regression equations, the calculated t-value was well below the critical t-value, indicating the intercept was not significantly different from zero. This suggests that there was no effect by factors not investigated (e.g., solvent, adsorption) on method accuracy.

Administration of Memantine

The addition of memantine hydrochloride (1.0 μM) to the perfusion medium resulted in arterial delivery and simulated systemic delivery. After the equilibration period of the eye, administration was achieved by switching from a drug-free perfusate to one containing memantine.

Direct administration was achieved by an intravitreal injection of memantine hydrochloride solution. Using a

syringe with 28 G needle (Beckton Dickinson) 250 µl memantine hydrochloride (5.5 mM) in PBS were injected to the centre of the vitreous body.

To simulate topical delivery, memantine hydrochloride was delivered by placing a ring (29 mm diameter) on the eye, covering the cornea and extending a few millimeter past the limbus to expose parts of the conjunctiva, thus creating a reservoir which was filled with drug solution. The memantine hydrochloride solution (9.27 mM) was prepared in borate buffer, containing sodium chloride (78.7 mM), boric acid (103.5 mM), and sodium borate (4.2 mM). The pH of the solution was adjusted to 7.4 with 1.0 M hydrochloric acid, as necessary. To completely cover the ocular surface enclosed by the ring, 4 ml of memantine HCl solution were placed in the reservoir for the total length of the perfusion experiment. At the end of the experiment the memantine solution was removed with a syringe and a blunt needle after which the tissue was carefully blotted dry with soft tissue paper.

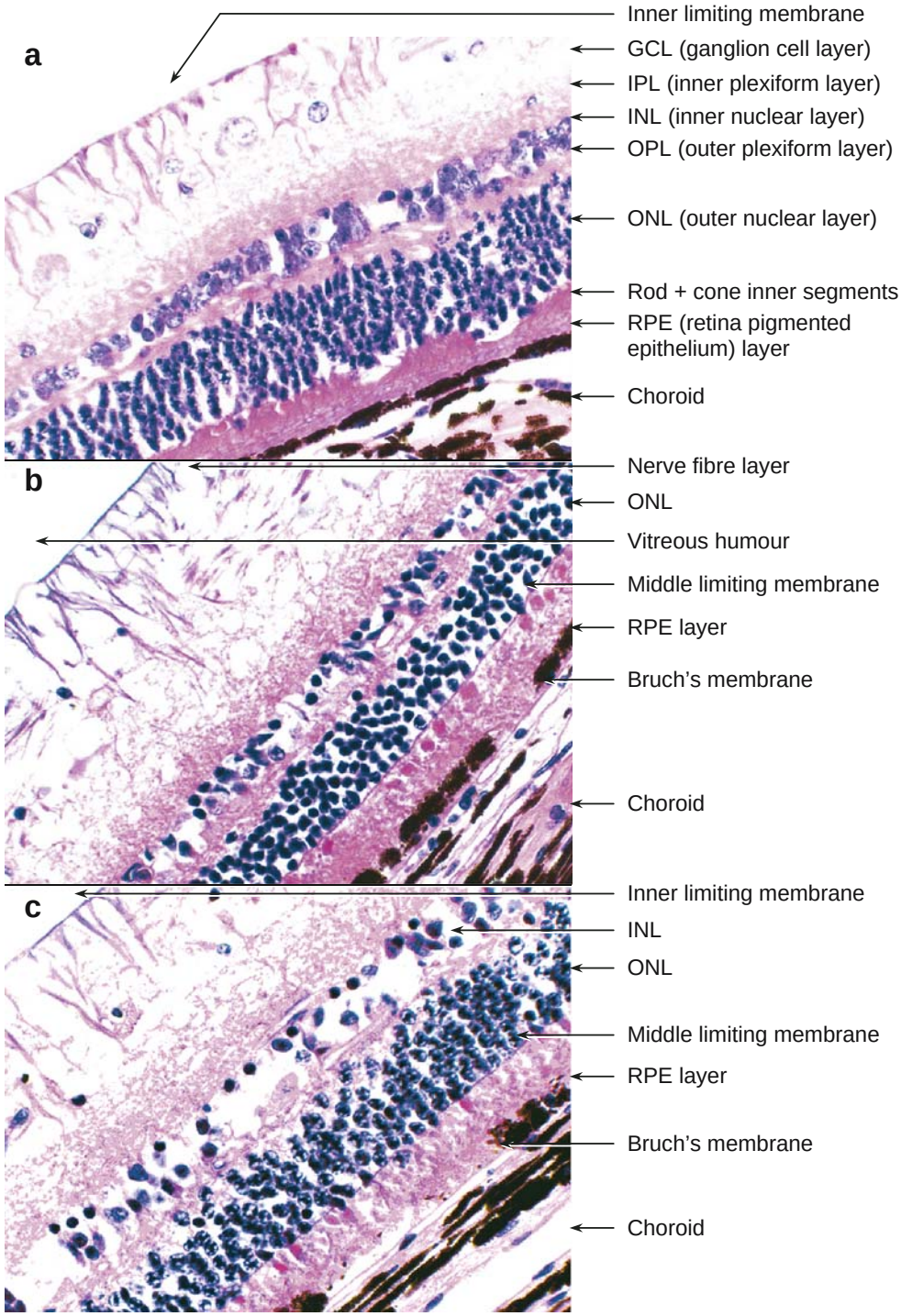

Fig. 3. Histopathology. Representative cross sections of bovine retinas. Hematoxylin and eosin (*H&E*) staining, light microscopy, 60× magnification. (a) No perfusion. (b) Perfused for 4.5 h. (c) Perfused for 9 h.

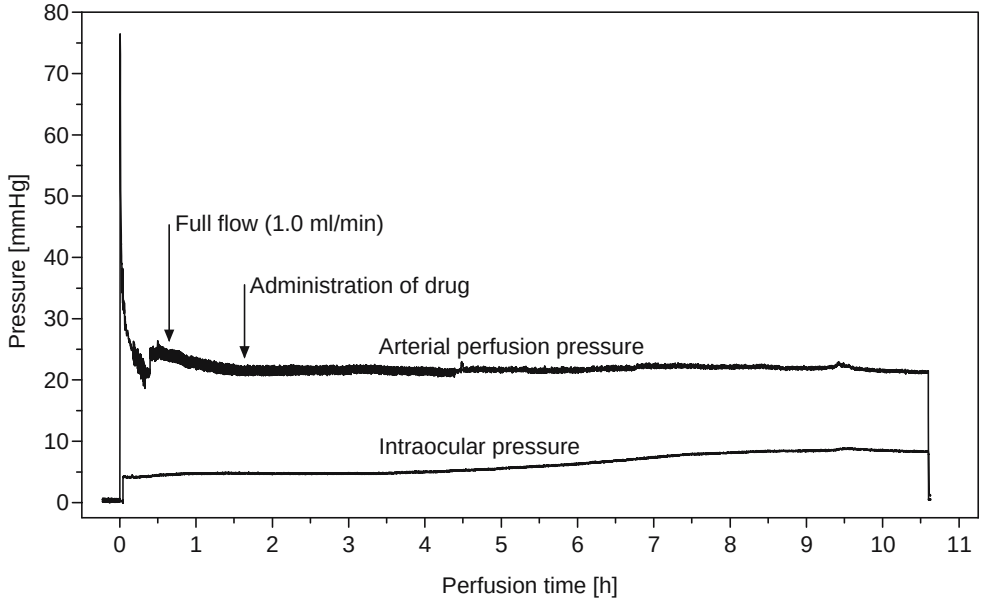

Fig. 4. APP and IOP. Recordings of arterial perfusion pressure (*APP*) and intraocular pressure (*IOP*). Initial pressure spikes may occur in the process of connecting the cannulated eye to the perfusion system during which the cannula may get bent and thus momentarily block the flow.

Tightness of the corneal ring against the eye was tested by quantifying memantine in the venous perfusate to which any leaked memantine solution would have been accumulated. Further, the volume of memantine solution removed at the end of perfusion was measured and was always only slightly less than the volume administered indicating tightness.

Dissection

On completion of the perfusion the bovine eye was removed from the perfusion chamber and the cannula and IOP measuring needle were withdrawn. A 25 G needle and 3 ml syringe (both Beckton Dickinson) were used to remove the aqueous humour which was transferred to a centrifuge tube. Adnexal tissue was trimmed off closely to the globe, remains of the extraocular muscles removed and the optic nerve cut at the entry to the globe.

The eye was then dissected into the different tissues (cornea, sclera, lens, iris-ciliary body (ICB), choroid/RPE, retina and vitreous humour) while care was taken to avoid cross-contamination. All dissected tissues were weighed (Table II) and stored in centrifuge tubes (15 or 50 ml, polypropylene, VWR) at −18°C until further processing.

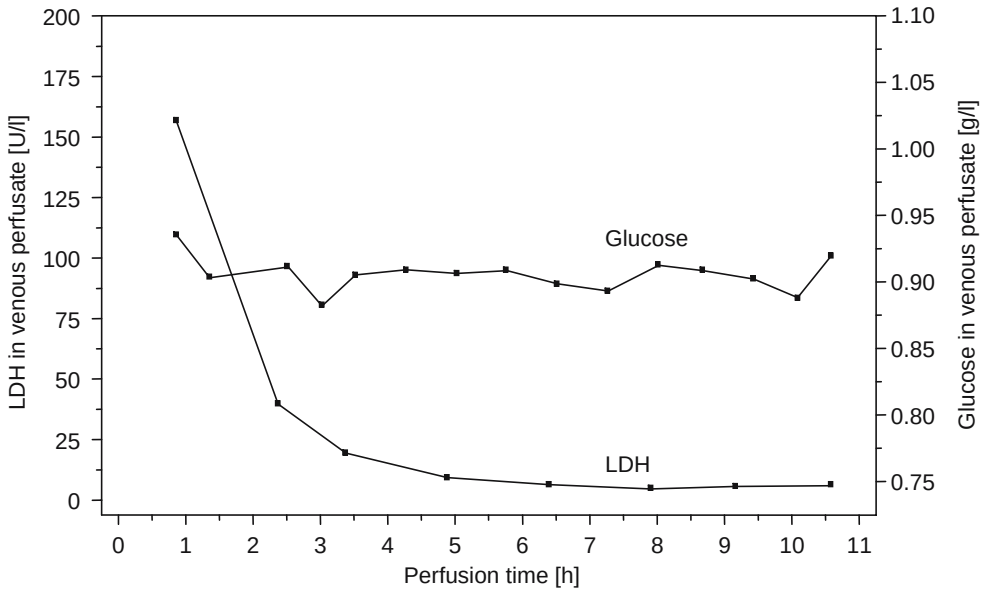

Fig. 5. Glucose and LDH. Glucose and lactate dehydrogenase (*LDH*) concentration in samples of venous perfusate collected at various times. The first sample was collected when flow of arterial perfusion was set to 1.0 ml/min (data of same eye as in Fig. 4).

Table I. Number of Successfully Perfused Eyes for Each Time Point

Perfusion time (h)	Intravitreal delivery	Systemic delivery	Local delivery
1	2	2	2
2	2	3	2
3	3	2	3
5	3	3	2
7	2	2	3
9	3	3	3
Total	15	15	15

Three eyes were perfused for each time point though some had to be removed due to impaired viability.

RESULTS

Histopathology

For the histopathological evaluation of the retina, freshly enucleated eyes (received within 2 h of slaughter) and eyes perfused for periods of 4.5 and 9 h were assessed for retinal lesions. For the assessment three eyes were perfused for 9 h, four eyes perfused for 4.5 h and a further four eyes were prepared without perfusion.

In these studies all eyes were prepared by slow injection of ~2 ml Davidson's fixative into one of the long ciliary arteries, followed by individual submersion in approximately 250 ml fixative for 24 h. The specimens were embedded in paraffin and 6 μm thick sections were stained with hematoxylin and eosin (H&E) for evaluation by light microscopy for lesions.

Cross-sections of tissue showed that the histology of the retina was either normal or minimally altered in freshly enucleated eyes which were not perfused (Fig. 3a). Eyes which had been perfused for 4.5 h (Fig. 3b) showed an intact Bruch's membrane. Retinal pigment epithelium (RPE) cells showed mild to moderate rounding and gaps were observed within the RPE layer. The photoreceptor layer (PRL) showed slight swelling and the outer (ONL) and inner nuclear layer (INL) were both minimally disrupted. Furthermore, a minimal loss of cells was observed in the ganglion cell layer (GCL) while the inner limiting membrane remained intact.

After 9 h of perfusion (Fig. 3c) the bovine eyes showed, in comparison to 4.5 h perfusion, increased rounding and loss of RPE cells with large gaps between them. Bruch's membrane remained intact and the PRL showed only slight swelling. The ONL and INL showed minimal and mild loss of cells, respectively. The loss of cells in the GCL after 9 h was moderate and the inner limiting membrane remained intact.

Viability Parameters

In order to assess the physiological status of the perfused eyes several viability indicating parameters were measured. If the following criteria were not met, eyes were excluded from the experiment.

APP measures the resistance of the eye to perfusion and is an indicator of the integrity of the blood-aqueous and blood-retinal barriers (35). While a viable eye generates a constant APP, any changes indicate deterioration of these barriers and thus compromised viability.

The inclusion criteria applied to the APP were that after an equilibration time the pressure should remain below 60 mm Hg and drift no more than 3 mm Hg/h. During initiation and equilibration of perfusion short periods of increased pressure were allowed since during this stage the cannula could be misaligned with the artery thereby blocking flow. The majority of eyes showed an arterial perfusion pressure in the range of 20 to 45 mm Hg. A representative recording of the APP over the course of a perfusion experiment is shown in Fig. 4.

A sustained IOP is an indicator of the secretion of aqueous humour by the ciliary process and its drainage by the trabecular meshwork (36,37). The trabecular meshwork is easily damaged and deterioration results in increased IOP. However, deterioration of the ciliary process is not reflected immediately by a decreasing IOP as the secretion of aqueous humour is primarily a filtration process.

The inclusion criterion applied to the IOP was that it has to remain below 10 mm Hg at any time during perfusion. The majority of eyes generated IOP values between 4 and 7 mm

Table II. Weight of Dissected Tissues of Freshly Enucleated (N = 23) and Perfused Bovine Eyes (N = 51)

	Fresh eyes (g)			Perfused eyes* (g)		
Tissue	Mean	SD	RSD	Mean	SD	RSD
Whole Eye	25.26	1.72	6.8	26.36	1.82	6.9
Aqueous humour	1.15	0.19	16.8	0.76	0.29	38.3
Vitreous humour	13.70	0.90	6.6	13.86	1.04	7.5
Cornea	0.57	0.05	9.2	0.64	0.14	22.1
Sclera	5.18	0.59	11.5	5.58	0.68	12.2
ICB	1.07	0.17	15.8	1.18	0.20	16.8
Choroid/RPE	0.44	0.15	34.3	0.78	0.22	27.6
Lens	1.78	0.15	8.3	1.85	0.11	5.9
Retina	0.74	0.06	8.1	0.87	0.09	10.3
Sum	24.62	1.65	6.7	25.53	1.79	7.0
Difference	0.64	0.24	38.3	0.83	0.22	26.5

*All perfused eyes met the viability criteria (eyes were also perfused without administration of memantine). Fresh and perfused eyes were dissected immediately upon receipt and following perfusion, respectively. All eyes were dissected in similar fashion and by the same person.

Table III. T_{max} and C_{max} Values

Tissue	Intravitreal Admin.		Systemic Admin.		Topical Admin.	
	T_{max} (h)	C_{max} (ng/g)	T_{max} (h)	C_{max} (ng/g)	C_{max} (ng/g)	T_{max} (h)
Retina	2	4,420	7	442	9	2,046
Vitreous humour	2	512	9	15	9	443
Aqueous humour	9	344	9	100	9	38,868
ICB	9	12,153	9	3,682	7	106,219
Choroid/RPE	7	6,943	9	1,451	9	3,894
Sclera	7	1,764	9	303	9	9,854
Cornea	5	180	9	45	2	39,769
Venous perfusate	2	113	9	109	7	163

Values were determined from the mean concentration for every time point. Intravitreal injecton 250 μl, 5.5 mM (297 μg); systemic delivery 1.0 μM, 1.0 ml/min (12.9 μg/h); topical reservoir 9.27 mM, 4.0 ml (8,002 μg/reservoir).

Hg. A representative recording of the IOP over the course of a perfusion experiment is shown in Fig. 4.

Glucose is the main source of energy for most cellular activities and is metabolised to a variety of compounds (38). Although cellular uptake of glucose is regulated primarily by insulin, cells have to be viable to facilitate uptake. Impaired cellular integrity reduces the transport of glucose across cell membranes, hence consumption decreases. However, glucose consumption shows relatively little sensitivity during tissue deterioration and indicates relatively advanced damage. For this reason, glucose consumption was used to monitor appropriate perfusion rather than tissue viability. During perfusion it is possible that the cannula punctures the artery thus stopping perfusion which may not be obvious. Therefore ocular perfusion was assured if the glucose concentrations of the venous perfusate were lower than in the perfusion medium. Representative measurements of glucose concentration in samples of venous perfusate over the course of a perfusion experiment are shown in Fig. 5.

LDH is an omnipresent cytoplasmic enzyme and is involved in the cellular metabolism of glucose. While LDH is confined to intracellular entities, damage to the plasma membrane releases the enzyme into the extracellular environment and thus venous perfusate (20,39,40).

Although LDH indicates tissue deterioration, the processes of enucleation and preparation of the eye for perfusion releases large quantities of LDH from extraocular tissues despite rinsing the prepared eye with perfusate. Thus, venous perfusate samples show initially high LDH activity which decline with time as the LDH is washed away from the external tissues. As a result, viability of the eye was taken to be compromised if LDH activities of two consecutive venous perfusate samples were higher than of the previous two samples.

Samples of venous perfusate for the determination of glucose consumption and LDH activity were collected at 30 min intervals for the first 5 h of perfusion and every 45 min thereafter. Representative measurements of LDH activity in

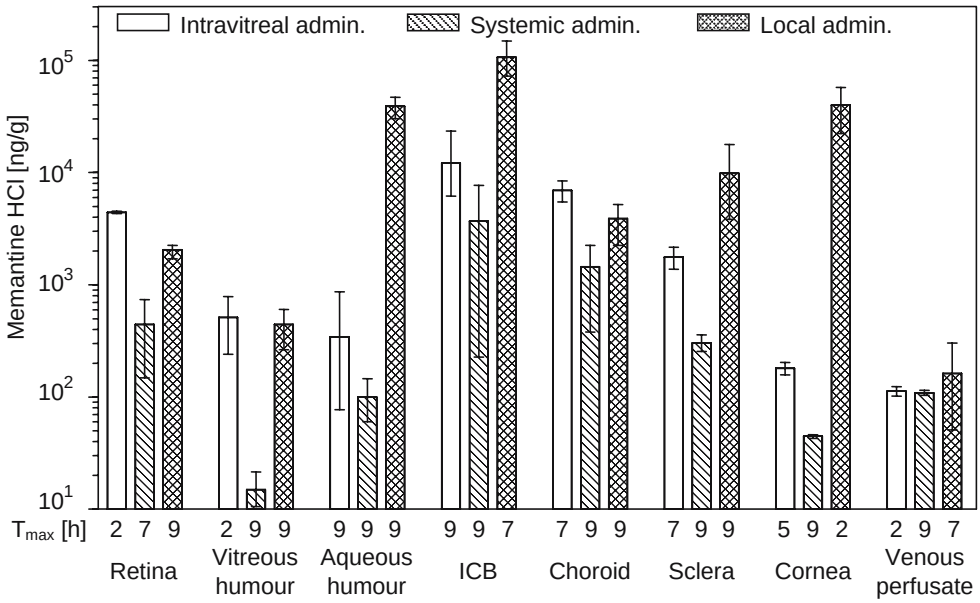

Fig. 6. Maximum concentration of memantine HCl (C_{max}) for the different tissues and routes of administration. Error bars indicate the minimum and maximum values observed at the time point of C_{max}. Intravitreal injection 250 μl, 5.5 mM (297 μg); systemic delivery 1.0 μM, 1.0 ml/min (12.9 μg/h); topical reservoir 9.27 mM, 4.0 ml (8,002 μg/reservoir).

samples of venous perfusate over the course of a perfusion experiment are shown in Fig. 5.

Number of Memantine Dosed Eyes Perfused

The number of perfused bovine eyes meeting the viability criteria described above are summarised in Table I. While three eyes were perfused for every time point some had to be excluded from further analysis, mainly because the IOP exceeded the upper limit of 10 mm Hg during the course of perfusion. Although some eyes had to be rejected due to poor viability, at least two eyes for every time point were successfully perfused, dissected and analysed. The tissue weights for the dissected tissues from non-prefused control eyes and perfused eyes are presented in Table II.

Tissue Concentrations

Concentrations of memantine HCl in ocular tissues after different lengths of perfusion for different routes of administration are shown in Figs. 7, 8 and 9. For the different ocular tissues the maximum concentrations (C_{max}) and the times (T_{max}) when these were determined are shown in Table III and Fig. 6.

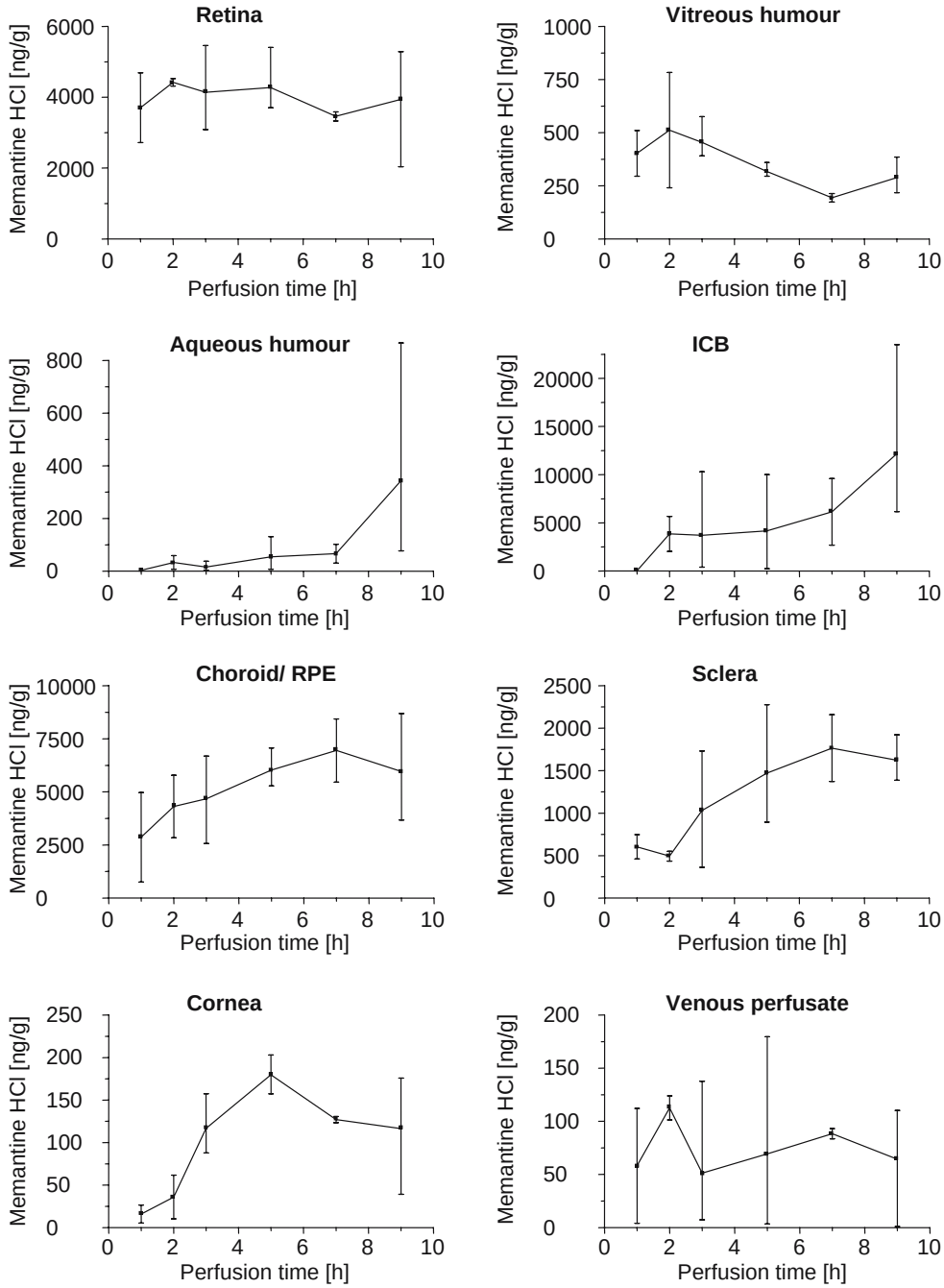

Fig. 7. Measured tissue concentrations of memantine HCl following a single intravitreal injection of memantine HCl (250 µl, 5.5 mM, 297 µg) in PBS. Error bars indicate the minimum and maximum values.

Tissue concentrations of memantine following intravitreal administration (Fig. 7) shows clearance from the vitreous humour and redistribution to other tissues. Memantine concentrations in the retina paralleled the vitreous levels indicating that memantine diffuses readily between these two tissues and steady-state is rapidly achieved. Retinal levels were consistently higher than the vitreous most likely due to its lipophilicity. The highest tissue concentrations were determined for the ICB and choroid/RPE which are located close to the site of administration. The cornea and aqueous humour had the lowest tissue concentrations.

Following vascular (systemic) administration, there was a varying but rising accumulation of memantine in each tissue (Fig. 8). The greatest tissue concentrations were observed for the melanin-containing ICB and choroid/RPE followed by the retina. The lowest concentrations were observed for the vitreous humour. Very low memantine concentrations were also observed in the avascular cornea and aqueous humor.

Tissue concentrations after topical administration to the ocular surface generally increased with perfusion time (Fig. 9). Tightness of the reservoir placed on the ocular surface was demonstrated by low concentrations of mem-

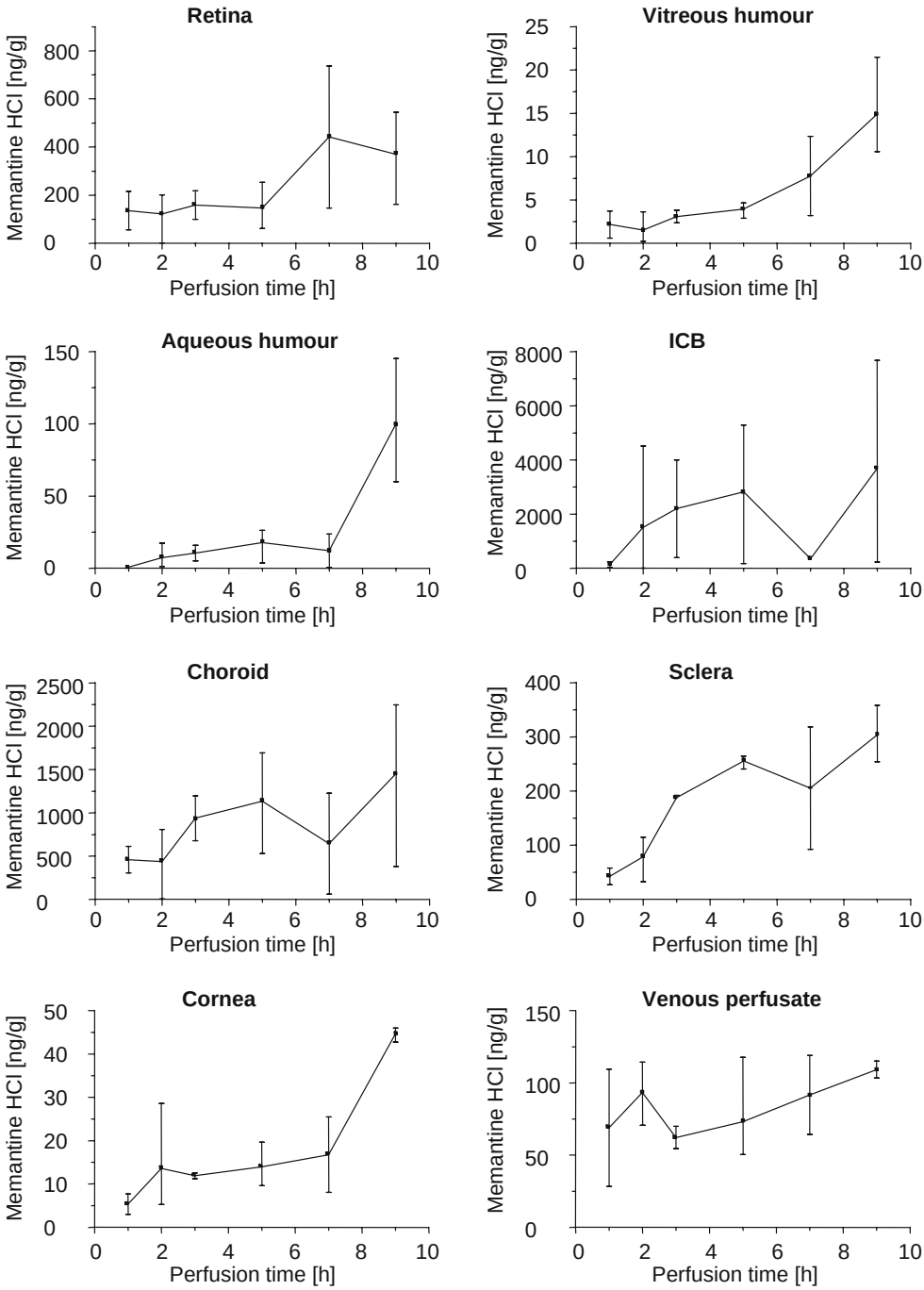

Fig. 8. Measured tissue concentrations of memantine HCl following systemic delivery of memantine HCl (1.0 μM, 1.0 ml/min, 12.9 μg/h) with the perfusate. Error bars indicate the minimum and maximum values.

antine in the ocular outflow (not shown). The cornea and sclera which were either in direct contact with the memantine solution or in close proximity showed a trend towards a plateau, indicating that tissues rapidly achieved a steady-state. While the highest tissue concentration of memantine was determined for the melanotic ICB, the lowest concentration was observed for the vitreous humour. However, memantine concentrations in the retina and vitreous were significant and increased with perfusion time.

DISCUSSION

A perfusion system was developed successfully which allowed the simultaneous perfusion of three bovine eyes at a constant flow rate. In addition, the system attempted to monitor the health of the eye by utilising parameters such as APP and IOP measured continuously. Other parameters including glucose consumption and LDH activity were analysed when perfusion was completed.

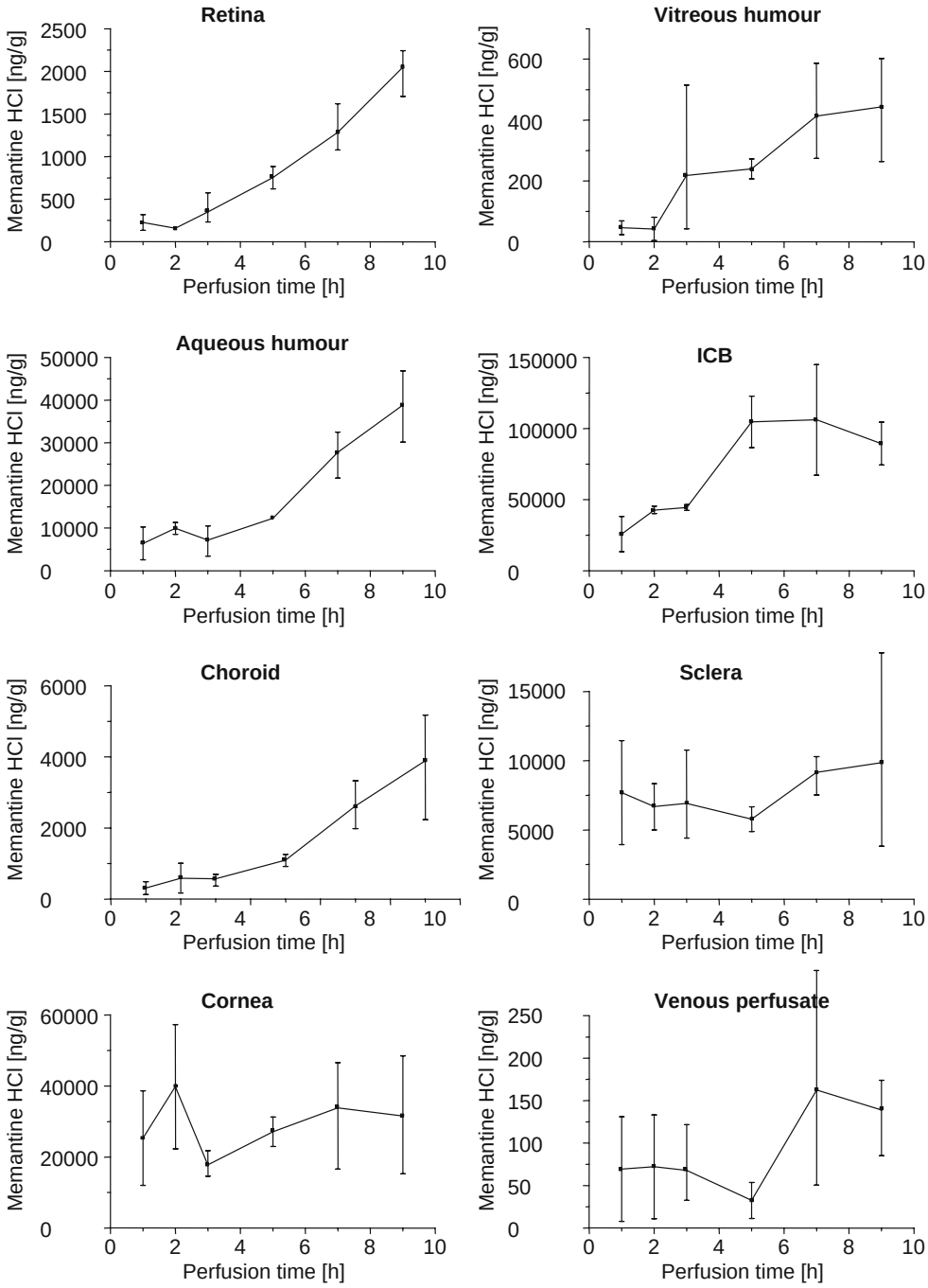

Fig. 9. Measured tissue concentrations of memantine HCl following topical administration of memantine HCl (9.27 mM, 4.0 ml topical reservoir, 8,002 μg/reservoir) in borate buffer. Error bars indicate the minimum and maximum values.

The combination of these parameters allowed an assessment of the appropriate perfusion and continuous integrity of the ocular structures for every perfused eye used in ocular experiments.

Histopathological examination of freshly enucleated eyes showed no or minor changes in the retinal structures. Perfusion for 4.5 h resulted in minor degeneration of some retinal substructures, particularly the RPE layer. Mild to moderate damage of retinal layers was observed after a perfusion period of 9 h.

Although bovine eyes deteriorate with perfusion time, all retinal layers remained well defined and recognisable throughout the maximum period of perfusion. Compared to freshly enucleated eyes, cell loss after perfusion for 9 h was considered to be below 20% in every retinal layer. Furthermore, the inner limiting membrane and Bruch's membrane showed no apparent damage. The preservation of the retinal structures and membranes suggests that it may be acceptable to monitor drug absorption and disposition for up to 9 h by this perfusion protocol.

Although by utilising the protocol described we have achieved a considerable increase in useful perfusion time, this period of viability may be extended even further. It is suggested that by minimising the time between slaughter and perfusion combined with the avoidance of temporary storage of the bovine eyes on ice, the equilibration time would be reduced which would substantially prolong the period of maintained viability and structural integrity. However, close co-operation between slaughterhouse and laboratory is paramount to achieve this goal.

While this perfused bovine eye model was primarily developed for pharmacokinetic studies with regards to ocular drug delivery, it may also find application in other areas such as drug metabolism, pharmacology and in the development of surgical procedures. For our purposes, the additional advantages of this model are that in comparison to animal studies it is relatively inexpensive, permits a high degree of control over experimental conditions and formulations may be used that could not be applied to live animals. Furthermore, this preparation permits simple administration of formulations by different routes including topical, intravitreal and with the perfusate.

Memantine was successfully administered to the model by intravitreal injection, with the perfusate and by topical infusion. While the drug-melanin interaction was investigated in a different study (29), the extent of binding suggested that this process may be of significance in the ocular disposition of memantine. These *in vitro* results were confirmed in this study by the accumulation of memantine in melanotic tissues in the arterially perfused bovine eye. Although the retina is the primary target site for memantine, the accumulation of it in melanotic tissues such as the ICB and RPE may act as accessory drug depots which would be beneficial for retinal delivery. Since the binding of memantine to melanin is reversible, therapeutic concentrations of memantine in neighbouring tissues such as the retina may be sustained over a prolonged period. Analysis of the memantine concentrations of the various tissues showed that memantine accumulated in melanotic tissues such as the ICB and choroid/RPE. In addition, memantine also accumulated in these tissues more rapidly.

Intravitreal administration to the perfused bovine eye showed that memantine diffused rapidly across the vitreous humour and that the retina is readily permeated. The memantine concentrations in the retina and vitreous paralleled each other indicating a steady-state had been achieved with respect to these tissues. Additionally, it appeared that memantine was eliminated from the vitreous body primarily by the posterior route. The low cornea and aqueous humor concentrations can be attributed to the route of memantine elimination following intravitreal administration. The high choroid/RPE and scleral levels indicate that memantine penetrates the RPE. Because of the enormous relative surface area of the retina compared to the retrozonular space, compounds that can penetrate the retina are cleared by a posterior route. The differential rates, a fast retinal clearance and slow diffusion to the aqueous humour, result in low aqueous humour and corneal memantine concentrations. Furthermore, accumulation of memantine in the ICB and choroid/RPE may also be due to melanin binding.

Following simulated systemic administration the highest tissue concentrations of memantine were found in the melanin-containing tissues followed by the retina and finally the vitreous showed the lowest concentrations. The vitreous is unstirred and as such poses a considerable diffusional barrier to drug absorption. This explains the large difference between the retinal and vitreal memantine levels. It is also important to consider the effects of perfusion in this model. The cannulated ciliary arteries perfuse the choroid and most ocular structures, however, the non-perfused central retinal artery provides blood flow to the inner retinal layer. While the retinal artery represents the inner blood-retinal barrier it is possible that perfusion of this artery would further increase retinal and vitreal memantine levels.

Topical delivery showed that memantine was able to penetrate to tissues of the posterior segment including the choroid/RPE and retina. High maximum tissue concentrations (C_{max}) were determined for the melanin-containing ICB and Choroid/RPE. While the cornea and aqueous humour, close to the site of administration, also showed high values for C_{max}.

Although the processes of the ocular disposition of memantine may be described by various pharmacokinetic techniques, the number of numerical parameters is great and may obstruct the immediate identification of important processes of ADME. We attempted to model the disposition observed for each route and for each tissue by numerical methods and whereas goodness of fit was excellent for vitreous, anterior chamber, sclera and corneal compartments, the tissue of most importance—the retina—eluded our attempts at a fit. After long consideration, we now appreciate this may be due to the important phenomena that interested us at the outset, the extensive melanin binding of memantine, which introduces strong influences into the disposition pattern. As an alternative, a visual approach which shows the disposition of memantine with perfusion time was suggested. For three sampling points, tissues of a cross-section of the eye were colour-coded according to the concentration of memantine HCl. The distribution and accumulation of memantine in various tissues, described earlier by compartmental and non-compartmental techniques, is shown in a visual fashion in Figs. 10, 11 and 12. While

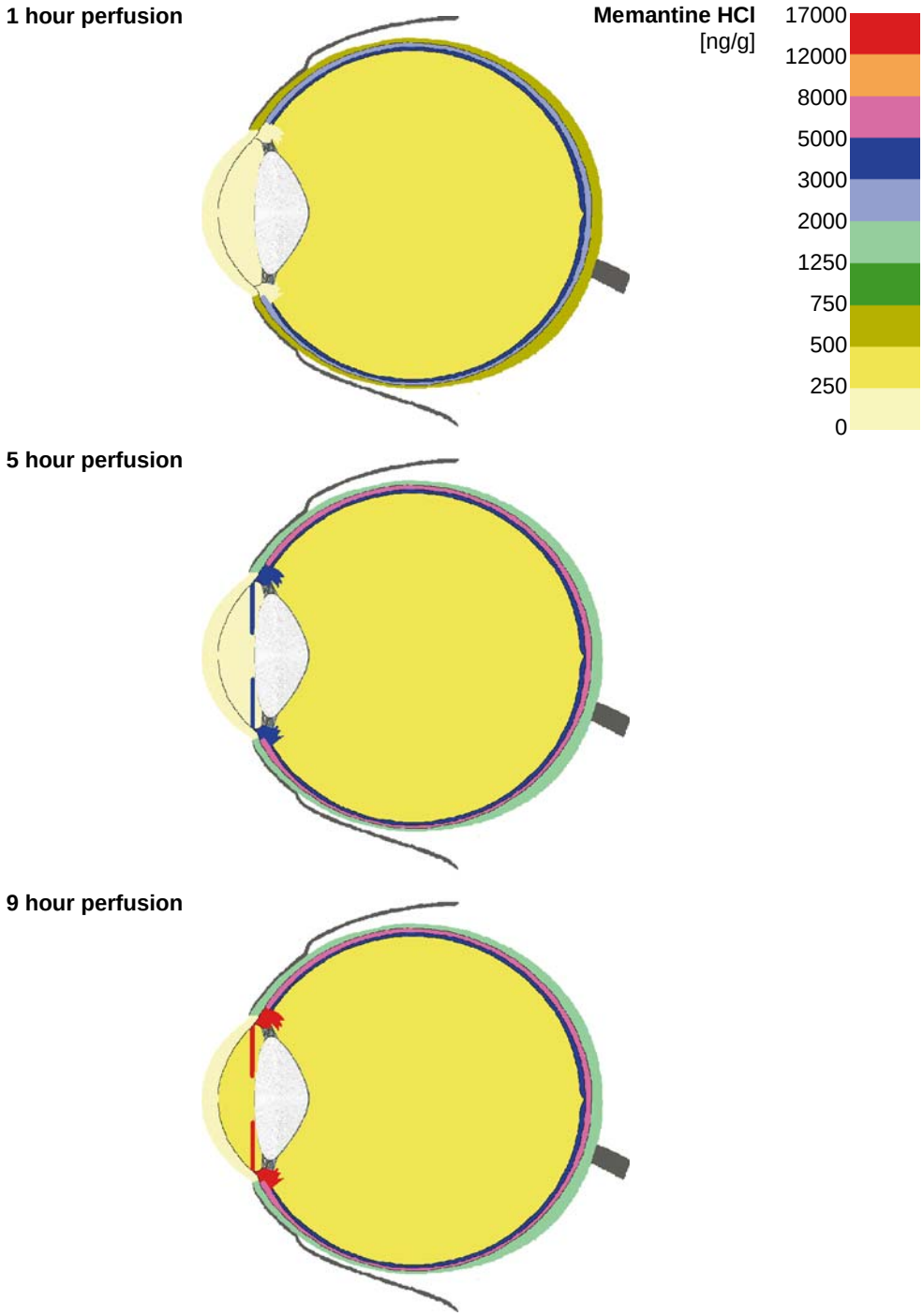

Fig. 10. Concentration of memantine HCl in various ocular tissues following intravitreal administration (250 μl, 5.5 mM, 297 μg). The lens, conjunctiva and optic nerve (coloured in *grey*) were not analysed for concentration of memantine HCl. The greatest concentration observed for this route of administration was divided into ten colour coded intervals to highlight the changes in tissue concentration during the course of perfusion.

the numerical techniques may be more precise, the visualisation of tissue concentration highlights the differences between the various tissues with regards to the accumulation of memantine with time and the effect the route of administration has on ocular disposition.

Although it was demonstrated that memantine reached the retina in considerable amounts from topical administration in this model, the practicality of it has to be investigated further. The drug solution holding reservoir, placed onto the cornea and parts of the conjunctiva, resembled principally topical infusion of memantine. Additional studies (data not presented) have verified that topically administered memantine achieves therapeutic retinal concentrations. Briefly, New Zealand white rabbits

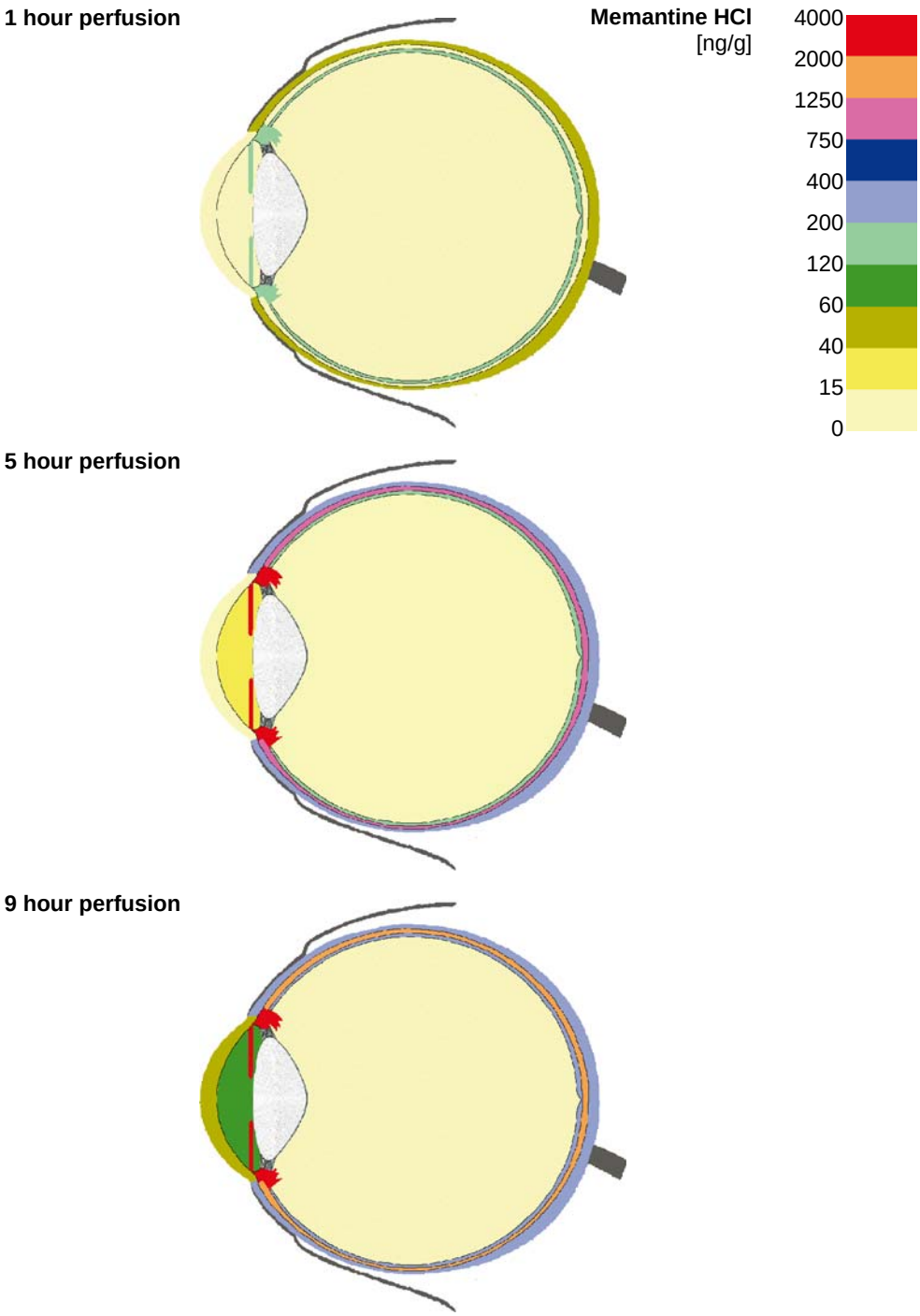

Fig. 11. Concentration of memantine HCl in various ocular tissues following systemic administration (1.0 μM, 1.0 ml/min, 12.9 μg/h). The lens, conjunctiva and optic nerve (coloured in *grey*) were not analysed for concentration of memantine HCl. The greatest concentration observed for this route of administration was divided into ten colour coded intervals to highlight the changes in tissue concentration during the course of perfusion.

were dosed topically with 35 μl of a 0.1% aqueous solution of memantine twice a day for 7 days. Another subset of rabbits was dosed orally with 2 mg/kg memantine for 7 days. The 2 mg/kg dosing regimen has previously been established as an effective dose for retinal neuroprotection. At the end of the dosing period ocular tissue concentrations were quantified. The retinal memantine concentrations from topical and oral dosing were essentially equivalent (107 ng/ml and 108 ng/ml, respectively). In preliminary studies on the relative ocular bioavailability of memantine, the drug was dosed once a day topically and daily orally for 1 week in New Zealand white rabbits. Drug concentrations in the untreated eye were generally low and indicated only a small amount of contralateral carry-over and that the

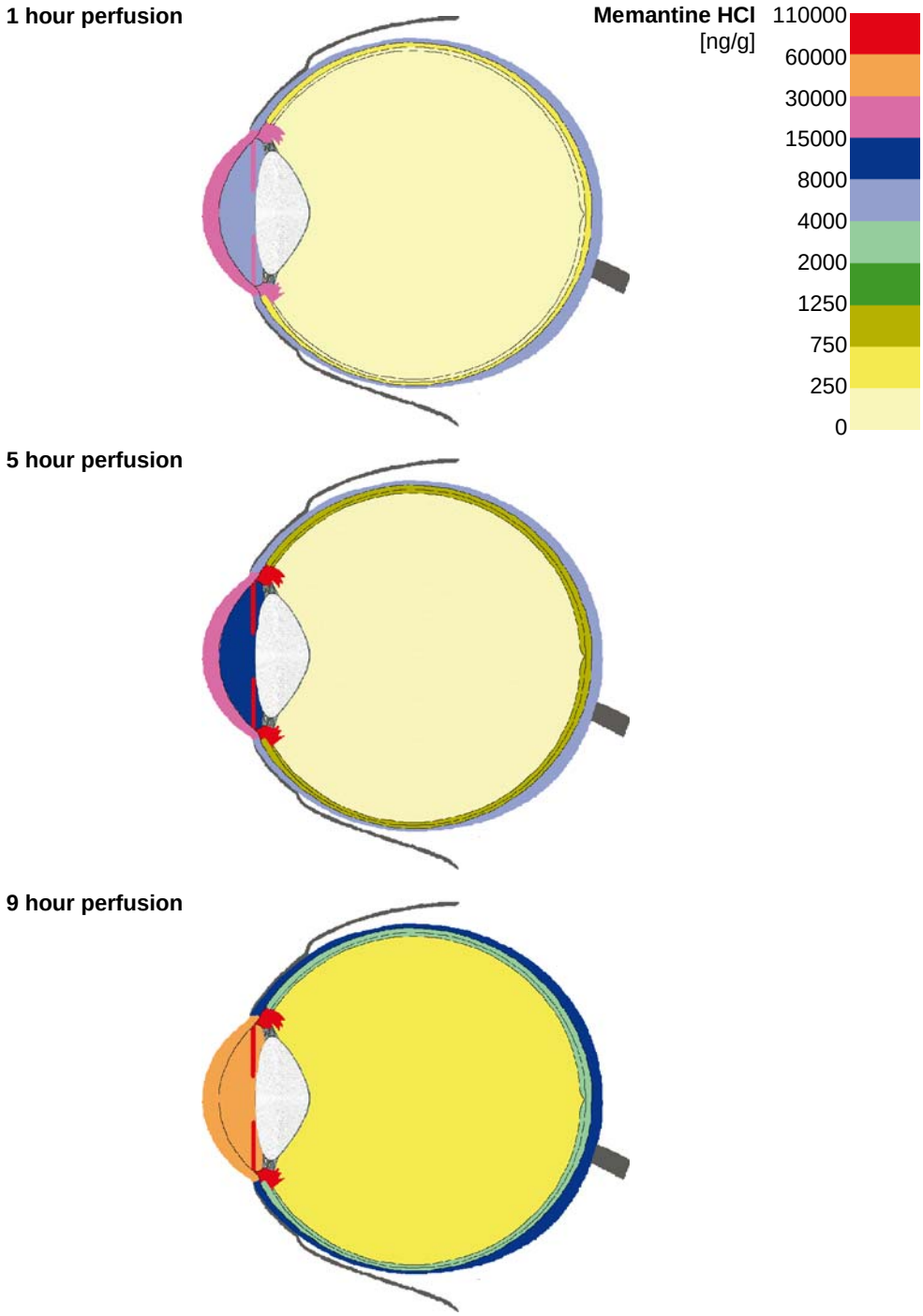

Fig. 12. Concentration of memantine HCl in various ocular tissues following topical administration (9.27 mM, 4.0 ml topical reservoir, 8,002 μg/reservoir). The lens, conjunctiva and optic nerve (coloured in *grey*) were not analysed for concentration of memantine HCl. The greatest concentration observed for this route of administration was divided into ten colour coded intervals to highlight the changes in tissue concentration during the course of perfusion.

ocular concentrations in the treated eye were from local penetration.

In previous research, we have also noted that [^{14}C]-memantine distributed to posterior segment tissues after topical instillation. Albino and pigmented rabbits were dosed via topical ophthalmic instillation with a 0.74% aqueous isotonic memantine HCl solution, pH 7.4. Autoradiographic data showed that memantine achieved significant concentrations in the posterior sclera, choroid and/or retina after topical dosing and that the intensity of the autoradiography was greatest in the pigmented tissues (41).

In addition to memantine HCl other compounds have recently been shown to achieve significant posterior segment concentrations from topical ocular administration. It has

been suggested by retinal and optic nerve head blood-flow measures that several adrenergic agents may achieve therapeutic posterior ocular concentrations after topical ocular drug delivery. These include timolol, betaxolol, latanoprost and dorzolamide (42–46). Dorzolamide was directly shown to achieve significant levels in the retina/choroid of pigmented rabbits after topical administration of a solution (47,48). The alpha-2 adrenergic agent brimonidine tartrate has also been directly shown to achieve therapeutic levels in the posterior segments of the eye after topical administration of 0.2 and 0.5% brimonidine tartrate ophthalmic solutions to cynomolgus monkeys (49–53). The vitreous concentrations of brimonidine after topical administration far exceeded the EC_{50} for activation of the alpha-2 adrenergic receptors (54). The vitreous penetration of brimonidine after topical ophthalmic administration has been confirmed in humans (55). Betaxolol and nipradilol also achieve significant concentrations in the posterior segment ocular tissues after topical administration (56,57).

In summary, it was demonstrated that the arterial perfused bovine eye system may be a useful model for the investigation of ocular drug disposition. It could be shown that memantine accumulated in melanin rich ocular tissues. Furthermore, it was shown that memantine achieved significant retinal concentrations in this model from all three routes of administration.

CONCLUSION

An arterial perfused bovine eye system was developed that maintained viability for 9 h. APP, IOP, glucose consumption and LDH activity were measured to ascertain appropriate perfusion and viability of each eye perfused. The arterial perfused bovine eye system proved to be a useful system for ocular drug delivery studies and has distinct advantages over experiments involving anaesthetized animals. The experimental results indicate that memantine will accumulate in the posterior segment when delivered by the topical route and that melanin-binding may support sustaining significant concentrations in the retina.

ACKNOWLEDGMENTS

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