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Magnetic Targeted Delivery of Dexamethasone Acetate across the Round Window Membrane in Guinea Pigs

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Abstract

Hypothesis—Magnetically susceptible PLGA nanoparticles will effectively target the round window membrane (RWM) for delivery of dexamethasone-acetate (Dex-Ac) to the scala tympani.

Background—Targeted delivery of therapeutics to specific tissues can be accomplished using different targeting mechanisms. One technology includes iron oxide nanoparticles, susceptible to external magnetic fields. If a nanocomposite composed of biocompatible polymer (PLGA), magnetite, and Dex-Ac can be pulled into and across the mammalian RWM, drug delivery can be enhanced.

Method—In vitro targeting and release kinetics of PLGA-magnetite-Dex-Ac nanoparticles first were measured using a RWM model. Next, these optimized nanocomposites were targeted to the RWM by filling the niche in anesthetized guinea pigs. A permanent magnet was placed opposite the RWM for 1 hour. Cochlear soft tissues, perilymph, and RWM were harvested after euthanasia and steroid levels were measured using HPLC.

Results—Membrane transport, in vitro, proved optimal targeting using a lower particle magnetite concentration (1 versus 5 or 10 mg/ml). In vivo targeted PLGA-magnetite-Dex-Ac particles had an average size of 482.8 ± 158 nm (DLS) and an average zeta potential -19.9 ± 3.3 mV. In 1 hour, there was significantly increased cochlear targeted delivery of Dex or Dex-Ac, compared with diffusion alone.

Conclusion—Superparamagnetic PLGA-magnetite-Dex-Ac nanoparticles under an external magnetic field (0.26 mT) for 1 hour significantly increased Dex-Ac delivery to the inner ear. The RWM was not completely permeated and also became loaded with nanocomposites, indicating that delivery to the cochlea would continue for weeks by PLGA degradation and passive diffusion.

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Keywords

Dexamethasone acetate; Magnetic targeting; Poly(lactide-co-glycolide); Round window membrane; Sensorineural hearing loss

Introduction

Sensorineural hearing loss (SNHL) is one of the commonest disabilities, globally affecting people both young and old. Estimates of up to 80% of individuals over 75 years of age have SNHL. In children and adults, if there is sudden trauma to the cochlea from excess noise, ototoxic substances, free radical release following ischemia-reperfusion, or autoimmune inner ear disease, then permanent hearing loss may be imminent. Delivery of therapeutic substances for hearing and balance disorders has been actively pursued over the previous 15 years^{1,2,3,4}. There is increasing evidence that steroids can treat these injuries within a window of time, which may prevent imminent loss of hair cells and preserve hearing. Treatment with anti-inflammatory glucocorticoid steroids is a common treatment today for sudden SNHL, typically administered by intratympanic (trans-tympanic membrane, IT) injection. This allows for subsequent uptake across the round window membrane (RWM) into the scala tympani with avoidance of potential side effects from long-lasting higher, systemic doses^{2,5}. More recently, treatment of Meniere's disease with IT injection of steroids has increased. Steroidal IT administration produces higher concentrations in perilymph and is superior to intravenous injection where little of the drug reaches the inner ear due to relatively low cochlear blood flow and the blood-labyrinthine barrier. Tight endothelial junctions and absence of fenestrae exclude larger molecules from filtering or diffusing from cochlear microcirculation⁶.

Dexamethasone (Dex) is a long-acting glucocorticoid steroid, with a half-life of approximately 4 hours⁷ and is cleared from the perilymph of guinea pigs with a three hour half-life⁸. These relatively short half-lives make dexamethasone a prime candidate for sustained release delivery, especially when targeted to the tissue site of therapeutic action. Magnetic targeted delivery may be capable of delivering dexamethasone nanoformulations relatively quickly and over a sustained period of time. In an effort to overcome the lack of constant contact of IT injectates with the RWM, a poloxamer based hydrogel containing micronized dexamethasone was evaluated IT in guinea pigs and showed high Dex perilymph levels and RWM exposure of up to 3 months⁹. We first proposed adding another dimension for cochlear drug targeting by pulling magnetically susceptible NP across the RWM using an external magnetic field¹⁰.

Poly D,L(lactide-co-glycolide) [PLGA] nanoparticles [NP] have been used to encapsulate and deliver different therapeutic payloads¹¹. PLGA is biocompatible, biodegradable, can be regulated for timed payload release, promoting intracellular delivery. The objective of this study was primarily to determine if targeted delivery of dexamethasone-acetate [Dex-Ac] could be achieved more efficiently across the mammalian RWM using magnetically-susceptible PLGA nanoparticles. We previously showed that Dex-Ac demonstrated better loading in PLGA than dexamethasone base or phosphate⁵. Magnetically targeting PLGA-magnetite-Dex-Ac nanoparticles was better than diffusion alone and the nanocomposite could cross the RWM more rapidly than diffusion and in a magnetically-force controlled manner, with potential release of Dex-Ac over subsequent weeks. Dex-Ac targeted to the cochlea exceeded experimentally therapeutic levels established previously³³. Consequently, better treatment of SNHL may be possible using magnetically targeted nanoformulations.

Materials and Methods

Animals

All animal experimental procedures, prevention of discomfort and pain, and euthanasia were conducted under approved protocol (# 08031) of the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center. Twenty four Hartley albino guinea pigs of both sexes were used, weighing 295–340 gm, supplied by Charles River Laboratories International, Wilmington, MA, USA.

PLGA-magnetite-dexamethasone acetate nanoparticle synthesis

Poly D,L(lactide-co-glycolide), 50:50: lactide/glycolide, was purchased from Absorbable Polymers International, (Pelham, AL, USA). Dexamethasone acetate and polyvinyl alcohol (PVA; MW 30–70 kDa), were purchased from Sigma Chemical Co (St. Louis, MO, USA). Superparamagnetic iron oxide nanoparticles [SPION] with oleic acid coating, 10–11 nm, were purchased from Liquid Research Ltd. (Bangor, Gwynedd, LL527 2UP, UK). Dichloromethane was purchased from VWR (Wester Chester, PA, USA).

An oil-in-water, single emulsion/solvent evaporation method produced Dex-Ac nanoparticles⁵. First, 200 mg of PLGA with or without magnetite (5mg/ml) was dissolved in 9 ml dichloromethane. Three ml of acetone containing 200 mg of Dex-Ac was then mixed with the dissolved polymer solution. The mixture was added to 40 ml of aqueous solution containing 2.5% PVA. The solution was sonicated on ice (Ultrasonic Processor VCX-130, Sonics, CT, USA) for 10 min at 40W and stirred for 4 hours to allow the organic solvent to evaporate. Particles were then isolated by centrifugation at 15,000 rpm (Beckman Optima LE-90K, Beckman Instruments Inc., Palo Alto, CA, USA) and washed three times with nanopure water (Millipore, Direct-Q, Billerica, MA, USA) to remove excessive drug, PVA or magnetite. Lastly, the particles were suspended in nanopure water, frozen, and lyophilized for 2 days and stored at –80°C.

Dex-Ac NP were weighed and dissolved in dichloromethane followed by extraction using methanol. The concentration of Dex-Ac was measured by HPLC with an Octadecyl C18 column; 5 micron bead size with column dimensions 4.6 × 150 mm (J.T. Baker, USA). The mobile phase consisted of phosphate buffer and methanol (1 g NaH₂PO₄·H₂O ACS Fisher, 700 ml methanol HPLC grade to 1000 ml with water). Dex-Ac quantity was calculated from a calibration curve made with samples having known Dex concentrations. Samples for the calibration curve were made by dissolving Dex-Ac in MeOH to make a 5 mg/ml solution followed by dilution hundreds of times with water to the final concentrations ranging from 1.25–20 µg/ml. The drug loading (DL) of the nanoparticles was calculated as follows:

$$DL(\%) = \frac{\text{weight of drug}}{\text{weight of drug} + \text{PLGA} + \text{SPION}} \times 100$$

Dex-Ac High Performance Liquid Chromatography Analyses of Cochlear Samples

Forty µL dichloromethane was added to freeze-dried samples and vortexed for 10 seconds, Then 160 µL methanol was added on and vortexed for another 10 seconds. This solution was centrifuged for 6 minutes and transferred to 100 µL vial for the autosampler where 20 µL was injected. The column was a Brownlee Analytical C18 5µm (length 150 × 4.6 mm (PerkinElmer, Santa Clara, CA, USA). The mobile phase was prepared with 1 g NaH₂PO₄ in 350 mL methanol diluted to 500 mL with water. Filtration to de-gas was performed on the day of analysis. The flow rate was 1 mL/min and we measured with UV at 242 nm. Maximum sensitivity at 0.002 AUFS was about 1 ng injected.

Experimental Designs

In vitro Magnetic Targeting in a RWM model

Our method of preparing an *in vitro* RWM model and testing magnetic targeting in this RWM model was described previously¹².

PLGA-magnetite-Dex-Ac Particle Preparation for in Vivo Experiments

Nanoparticle solutions used for the guinea pig targeting experiments were prepared by placing 2 mg of PLGA-magnetite-Dex-Ac in either 0.5 or 1 mL of nanopure water (1 or 2 mg/mL). The solutions were kept on a cold pack during the surgical procedure to minimize PLGA release and maintain solution stabilization. If particle agglomeration was observed the solution was sonicated for 5–10 seconds (Misonix XL 2000, Farmingdale, NY, USA) to resuspend them. A particle-positive control was collected in order to calculate the amount of drug in the dose that was given to each animal.

Stereotaxic Surgical Technique

Consistent head position of guinea pigs for magnetic targeting was surgically important because magnetic nanoparticles move vertically down magnetic gradients toward the magnet pole face. Hence, horizontal orientation of the RWM niche was necessary for vectoral magnetic targeting, through the RWM. Since it is not possible intra-operatively to vertically observe the guinea pig RWM niche (diagonal microscopic visualization is used operatively), it was necessary to determine a head position that horizontally positioned the niche. This was accomplished using a Laser Triangulation Sensor (Model Microtrack II, MTI Instruments, Albany, NY, USA) and a separate set of 3 guinea pigs. The niche was first exposed to reveal all margins. The Sensor, sensitive to microns, measured distances to the niche perimeters while manually adjusting the animal heads. It was determined that when the angle of the head was rotated 51 $\pm$ 2 degrees to the left and the nose of the animal tilted downward 2–4 degrees, that the niche was optimally positioned for filling the niche with NP solutions. A custom plastic platform was built to position the guinea pig RWM at horizontal zero during targeting experiments.

After weighing, anesthesia was induced by (i.m.) injection of 80 mg/kg of ketamine HCL (Bioniche Pharma LLC, Lake Forest, IL, USA) and 10 mg/kg of xylazine (RX Veterinary Products, Westlake, TX, USA). The operative site was infiltrated (s.c.) with 0.5 ml of 0.5% lidocaine with epinephrine, 1:200,000 U/ml (Hospira Inc, Lake Forest, IL, USA). Anesthesia was maintained with supplemental one-third doses. A right post-auricular incision was made and using an operating microscope (Zeiss, OP MI 1, Karl Zeiss, Thornwood, NY, USA), a 2–3 mm fenestration was made in the bulla using a 1.5 mm diamond burr (Medtronic Legend Drill System, Medtronic ENT, Jacksonville, FL, USA). The bony cochlear ledge overhanging the RWM was reduced using a 1 mm diamond burr and the animal was placed on the plastic platform to position the RWM over the pole face of a custom rare earth (NdFeB; 0.26T) 4 in. cube magnet (Integrated Magnetics, Culver City, CA, USA).

Using a 5 μ l syringe (Model 7105, Hamilton Syringes, Reno, NV, USA), the RWM niche was filled with the NP solution (0.4 μ l) and the animal was placed, operated ear up, with RWM parallel to the pole face of the magnet for 30 min. The animal was removed, and paper points used to gently wick away the remaining NP solution. A second 0.4 μ l dose of NP solution was microinjected into the niche for another 30 min. of targeting. Matched control animals received the same 2 $\times$ 30 min. dosing, but without magnetic targeting.

Following a double anesthetic dose, animals were decapitated and both bullae removed. Each temporal bone was sealed in paraffin wax and the bullae refrigerated until cochlear

dissection. Harvesting of perilymph, cochlear soft tissues and RWM subsequently was conducted under a dissecting microscope. A 0.5 mm cochleostomy was made in the basal turn and perilymph was aspirated from the scala tympani using a Hamilton syringe (10 μ l, Hamilton Company, Reno, NV, USA). The cochlear perilymph space was rinsed 3–4 times with 10–15 μ l nanopure water through an apical cochleostomy. Fluid collected from the basal turn, the RWM plus niche and soft tissues (stria vascularis, spiral ligament and modiolar tissues) were saved in separate microcentrifuge tubes. The three samples from each cochlea were stored at -20°C until HPLC analyses.

Statistical Analyses

For the in vitro experiments, results of the control and magnetic targeting were expressed as means of six experiments $\pm$ SEM. The comparison was performed by using one-way ANOVA (SPSS 14.0 for windows) and a post hoc test (Tukey HSD). For in vivo studies, comparisons between the groups were made using the Student's t-test, and with ANOVA in conjunction with the Holm-Sidak method, for control group versus experimental groups. A value of $p < 0.05$ represented a statistically significant difference between the groups. Data groups were presented in terms of a mean and a standard error of the mean (SEM), which describes the level of uncertainty between sample means and population means. Data were analyzed using SigmaStat for Windows v3.11 (Systat Software Inc., Chicago, IL, USA).

Results

Nanoparticle Characterization

Hydrodynamic size of the nanoparticles (DLS) and their zeta potential were determined using ZetaPlus (Brookhaven Zeta PALS, Holtsville, NY, USA). PLGA-Dex-Ac-magnetite particles used in animals averaged 482.8 ± 158 nm with an average zeta potential of -19.9 ± 3.3 mV. The nanoparticles imaged using scanning electron microscopy (JEOL JXM 6400 Scanning Electron Microscope, JEOL USA, Inc. Peabody, MA) are shown in Fig. 1.

Our previous magnetic characterizations of PLGA-magnetite particles¹³ as well as in the present study using Vibrating Sample Magnetometry (DMS Model 1660, USA) showed an average magnetic susceptibility of 8.69 electromagnetic units/gm for 4 repeat VSM runs (Fig 2). Multiple superparamagnetic magnetite NP (10–11 nm) were encapsulated in each PLGA nanocomposite (451 nm).

In vitro Nanoparticle Release Kinetics

Limited solubility of Dex-Ac meant that release was controlled by conversion to more soluble dexamethasone. One release kinetics experiment dissolved 28.2 mg of particles in 150 mL of water. Samples taken at 0, 1.25, 4.2, 10.3, 17 and 37 days were assayed for Dex and Dex-Ac by reverse phase HPLC with UV detection. At 37 days the total concentration (Dex equivalent) was 71 $\mu\text{g/mL}$, equivalent to 37.8% of particle weight. Five experiments on 5 samples in 45 ml were collected over 26 days and assayed for Dex, Dex-Ac and 2–23% of the particle weight was released after 26 days. The NP tested (508 nm diameter; -21 mV) had a Dex content of 45.3 % (Fig. 3).

In vitro RWM model Magnetic Targeting

Our in vitro RWM model demonstrated that transport of PLGA-magnetite-coumarin nanocomposites, based on coumarin fluorescence detection, varied inversely with magnetic susceptibility (related to the magnetite concentration PLGA). We verified that PLGA-magnetite-coumarin nanoparticles synthesized using 1 mg/ml magnetite yielded a three-fold increase in transport compared to no magnet. Note the two-fold increase seen in transport of NP synthesized using 5 mg/ml magnetite concentration (Fig. 4).

Magnetic Targeted Delivery to the Inner Ear

Intra-operatively we observed visually that some of the particles were accumulating in and on the RWM in a boundary layer⁴. Since those NP eventually would deliver the steroid, we analyzed RWM, perilymph, and cochlear soft tissues for both Dex and Dex-Ac. Magnetic force tends to pull particles straight through RWM and into scala tympani soft tissues, so these were scraped from the cochlea and assayed using HPLC. Figure 5 shows the averaged ($\pm$ S.E.M) individual tissue analyses for Dex plus Dex-Ac in the three tissue compartments in eight animals. Because of high variances, the only statistically significant increase was in perilymph, though increased delivery was found in all three compartments. When those tissues were combined (Fig 6), there was significantly increased targeted delivery compared to diffusion alone across the RWM.

Discussion

Dexamethasone was first studied for treatment of the inner ear by Juhn¹⁵. Multiple, non-systemic delivery systems for anti-inflammatory and immunosuppressive steroids, dexamethasone being preferred, previously have been tested. Newer technologies utilized hydrogels and nanoparticles^{2,3,4,16,17,18}. There has been a shift toward targeting cochlear tissues only, with approaches including: a) IT injection (filling) the middle ear by single or repeated doses, along with various solutions or transport facilitators, such as alginate, hydrogels, gelatin, hyaluronic acid, or chitosans^{4,19,20}; b) infusion into or near the RWM niche using a microcatheter device²¹; c) wick insertion for infusion through TM ventilation tubes³; d) a microfluidics-based perfusion device²²; e) nanoparticle uptake and diffusion across the RWM^{1,18,23}; and f) our recent method of magnetic targeting^{10,12}. Limitations remain for cochlear targeting through the RWM. Because of variations in techniques, therapeutic substances, doses and small numbers in trials, there remains no clinically conclusive statement about the efficacy of IT steroids treating SNHL. Three randomized clinical trials on patients with SNHL reported benefits from IT steroid therapy as a salvage treatment when systemic injections failed⁴.

A hurdle of magnetically targeting the cochlea through RWM is that it is not highly permeable and is a protective barrier. The average human RWM is about 70 μ m thick. The outer epithelial layer with tight junctions appears to be “permeation defensive”²⁴. Blood vessels present in the RWM middle connective tissue layer can pick up NP and carry them away in the systemic circulation (Dormer et al. unpublished ^{99m}Tc biodistribution data). The inner epithelium has loose junctions and a discontinuous basement membrane. Nevertheless, cationic microspheres had been shown to pass from the scala tympani into the RWM. Diffusion or transport across this tripartite RWM requires varying times and the amount of molecular substances that can pass through depends on size, stoichiometry, lipophilicity, electrical charge and concentration²⁴. Additionally, IT injection volumes must remain in contact with the RWM for diffusion to occur. Hence with IT delivery, drainage from the Eustachian tube, absorption by middle ear mucosal cilia, and gravitational settling all reduce available drug for RWM diffusion. Also, there are anatomical indications that the RWM behaves as a semipermeable membrane, only allowing uptake of select substances from the middle to inner ear^{15,25}. At the same time, the RWM primarily appears as an anatomically protective barrier against substances moving from the perilymph into the middle ear space. In summary, it appears the RWM is secondarily semi-permeable while it primarily serves as a compliant membrane for cochlear fluid dynamics. For these reasons external magnetic forces on NP can facilitate, as we have shown, RWM transport of Dex-Ac. Numerous therapeutic and carrier materials have been reported to enter the perilymph from the middle ear cavity by diffusion alone³ and therefore diffusion might be both be enhanced and able to be quantitated by magnetic targeting.

Biodegradable polymers are being investigated for targeted drug delivery as they do not require subsequent removal after delivering therapeutic payload(s). The co-polymer PLGA is the most commonly used medical polymer worldwide^{26,27}. Nanoformulations of PLGA are produced by oil-water double emulsion, high pressure homogenization or spray drying²⁸. For specific targeted therapies, respective biodistributions, safety and toxicity must be documented before clinical approvals. Nanocomposites have been investigated as encapsulants for chemotherapeutics²⁹, anesthetics, antihypertensive agents, immunomodulators, hormones, genes, nucleic acids³⁰, proteins, peptides and antibodies²⁶. PLGA nanoparticles have been targeted to lung, brain³¹ and the cochlea^{1,32,33}. An earlier study combined PLGA with Dex for pretreatment of brain electrodes to preclude glial inflammation³⁴. Dexamethasone was released up to three weeks, following an initial burst release. Only one group had previously explored PLGA as a NP vehicle for the inner ear¹. They compared a siloxane-polymer-gelatin hydrogel NP with PLGA-encapsulated beclomethasone. We hypothesized that if PLGA-magnetite-Dex-Ac could be optimized, magnetic forces would enhance crossing the RWM for delivery of Dex-Ac into the mammalian cochlea.

Dexamethasone IT administration results in significantly higher perilymph concentrations than intravenous administration^{6,35}. Dex- loaded lipid NP or a poloxamer based hydrogel containing micronized Dex (IT) provided sustained-release of Dex in the middle ear cavity. However, clearance of the drug from the middle ear cavity or lack of continuous contact with the RWM will decrease the efficiency of drug delivery across the RWM^{9,36}. We first proposed adding another dimension for cochlear targeting, pulling magnetically susceptible NP across the RWM using an external magnetic field, would target the inner ear better than RWM diffusion¹⁰. Of the Dex, Dex-Ac in 0.8 μ l of solution placed in the RWM niche, 10% was measured as delivered, reaching 18 ng/ μ l in 60 min., exceeding the experimentally shown Dex therapeutic index of 0.03–0.04 ng/ μ l³³. Delivery effectiveness (perilymph concentration divided by peak delivery time) of 0.3 ng/ μ l/min represents the best ever reported^{8,9,19,33,36}. To the best of our knowledge, the only Dex or Dex-Ac delivery method reporting higher delivery effectiveness was Hahn³⁷ in 2006, by continuously infusing high concentrations of Dex-dihydrogen phosphate disodium salt directly onto RWM. They reported 1.5 ng/ μ l/min., however their perilymph concentrations measured by dialysis had very large variance (291 $\pm$ 369 ng/ μ l). This delivery mode would be very difficult to translate to the clinic. Magnetic targeting, on the other hand, causes NP to cross the RWM with time-dependent and force –dependent parameters, partially remaining in the niche, maintaining contact with the RWM, where they can provide sustained-release for days to weeks as particles diffuse across the RWM and PLGA biodegrades. PLGA-magnetite-Dex-Ac NP pulled into and onto the RWM could more readily be translated to ambulatory patients.

Our approach used three principles to improve targeted intracochlear delivery of Dex-Ac through the RWM. First, the therapeutic solutions were topically placed only, directly into the RWM niche. Secondly, the Dex-Ac was encapsulated in PLGA, which concentrated, protected and dispersed steroid payload. Thirdly, magnetic targeting added force for RWM penetration. Generally, clinical and basic science data indicate that higher local levels of drug occur with targeted, as opposed to systemic, administration. Our data demonstrate a further two-fold enhancement of Dex-Ac delivery to the cochlea with magnetic targeting compared to diffusion. Theoretically, advances in magnetic targeting may induce even faster, more complete and higher doses of PLGA-magnetite-Dex-Ac NP into RWM, perilymph, and cochlear tissues. Nevertheless, animal to animal variability remained high with this model using a much smaller niche than in the human, hence dosage control and quantitation would benefit from additional studies. Several areas can be improved to deliver more drugs into the inner ear in the future: 1) Nanoformulations may be modified to

decrease agglomeration of particles on RWM surface; 2) Transport-facilitating agents, (e.g. histamine, chitosan) may be added to increase RWM permeability⁶; and 3) Nanoparticle shape changes (e.g. cylinders, rods, wires) may improve RWM penetration.

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Figure 1.
Scanning Electron Micrograph image of DEX-Ac incorporated PLGA nanoparticles.

Figure 2.

Magnetic hysteresis curves showing the magnetic susceptibility of PLGA-magnetite nanoparticles in 4 runs of a Vibrating Sample Magnetometer. Average susceptibility of 8.69 emu/gram is not large but sufficient to increase targeting to the cochlea.

Figure 3.

Release of dexamethasone and dexamethasone acetate from nanoparticles containing dexamethasone acetate over 37 days. Left y-axis represents concentrations dissolved and the right y-axis represents the amount of dexamethasone and acetate weight per weight of particles released versus time.

Figure 4.

RWM magnetic targeted delivery of CMNPs with low magnetic susceptibility. Results are expressed as means $\pm$ SEM. Student's paired two-tailed t-tests was used. Asterisks *, **, *** represent $p < 0.05$, < 0.01 , < 0.001 , respectively. RWM, round window membrane; CMNPs, coumarin labeled magnetic nanoparticles.

Figure 5. Dex + Dex-Ac in Perilymph, Round Window Membrane, and Soft Tissue

Average amounts (ng) of Dex plus Dex-Ac detected using HPLC in 8 guinea pigs following 1 hr. of magnetic targeting across the RWM.

Figure 6. Dexamethasone/Dexamethasone Acetate Delivery Across the Round Window Membrane

Average amounts (ng) of Dex plus Dex-Ac detected in 3 compartments combined of RWM, perilymph and scala tympani soft tissues in 8 guinea pigs following 1 hour of magnetic targeting across the RWM.