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Reflux-free cannula for convection-enhanced high-speed delivery of therapeutic agents

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Abstract

Object—Clinical application of the convection-enhanced delivery (CED) technique is currently limited by low infusion speed and reflux of the delivered agent. The authors developed and evaluated a new step-design cannula to overcome present limitations and to introduce a rapid, reflux-free CED method for future clinical trials.

Methods—The CED of 0.4% trypan blue dye was performed in agarose gel to test cannula needles for distribution and reflux. Infusion rates ranging from 0.5 to 50 μ l/minute were used. Agarose gel findings were translated into a study in rats and then in cynomolgus monkeys (*Macaca fascicularis*) by using trypan blue and liposomes to confirm the efficacy of the reflux-free step-design cannula in vivo.

Results of agarose gel studies showed reflux-free infusion with high flow rates using the step-design cannula. Data from the study in rats confirmed the agarose gel findings and also revealed increasing tissue damage at a flow rate above 5- μ l/minute. Robust reflux-free delivery and distribution of liposomes was achieved using the step-design cannula in brains in both rats and nonhuman primates.

Conclusions—The authors developed a new step-design cannula for CED that effectively prevents reflux in vivo and maximizes the distribution of agents delivered in the brain. Data in the present study show reflux-free infusion with a constant volume of distribution in the rat brain over a broad range of flow rates. Reflux-free delivery of liposomes into nonhuman primate brain was also established using the cannula. This step-design cannula may allow reflux-free distribution and shorten the duration of infusion in future clinical applications of CED in humans.

Keywords

convection-enhanced delivery; step-design cannula; reflux; central nervous system; liposome; *Macaca fascicularis*; rat

CONVECTION-ENHANCED delivery, which makes it possible to distribute large volumes of therapeutic agent to target regions in the CNS, is a promising approach in treating various diseases of the CNS that respond poorly to systemic chemotherapy or surgical treatment. Convection-enhanced delivery is a direct intracranial drug-transfer technique that uses a bulk-flow mechanism to convey and distribute macromolecules to clinically significant volumes of solid tissues.^{2,17,23} Compared with simple diffusion procedures, CED enables a

greater volume of distribution and is designed to direct a drug to a specific target site. It bypasses the blood–brain barrier, which is a major obstacle for many systemically applied drugs.² Several authors have performed extensive studies in an effort to adapt CED for clinical use.^{1,3,6–9,11,14,15,17–19,22,23}

Recently, CED has been used in clinical trials in patients with recurrent glioblastoma multiforme^{12,13,16} and in parkinsonian patients to deliver glial cell line-derived neurotrophic factor.⁸ Note, however, that the lack of a standardized cannula design, a low infusion rate, and reflux of the delivered agent are major barriers to broad clinical use of CED at present. Cannulas now used in clinical trials have a large diameter,¹³ permitting only a low flow rate to minimize reflux.^{2,3,13,14,17} This low infusion rate is associated with a long duration of treatment, which exposes patients to a greater risk of infection and to an extended period of physical discomfort and emotional stress. Lidar, et al.,¹³ reported the onset of chemical meningitis during and wound dehiscence after CED due to drug leakage in a clinical trial of paclitaxel for treatment of malignant gliomas. Thus, side effects caused by the reflux of administered agents may prevent safe delivery of a therapeutic agent through CED. We hypothesized that the development of a reflux-free cannula would improve the efficacy of CED and that minimizing reflux while infusing larger volumes at a higher flow rate would achieve more effective convection, which may improve the therapeutic index of CED.

In animal experiments, Morrison and colleagues¹⁷ have shown that reflux can be decreased and CED enhanced by reducing the diameter of the needle used from 27 to 32 gauge. Nonetheless, even the 32-gauge needle, one of the smallest metal needles commercially available, must be used at a flow rate of 0.5 μ l/minute to avoid reflux.^{7,14,17} We created a new, reflux-free, step-design cannula that permits CED at a higher flow rate.

Cannulas presently used for CED were tested for distribution and reflux at various infusion rates, and new designs were developed initially through CED of trypan blue dye in agarose gel, which has previously been described as a reliable model to simulate CED in in vitro brain.^{4,5,10,20,21} Agarose gel findings were translated into a study in the rat brain and then in the nonhuman primate (*Macaca fascicularis*) brain by using trypan blue and liposomes to confirm the efficacy of the reflux-free step-design cannula in vivo.

Materials and Methods

Reflux-Free Step-Design Cannula

First, metal cannula needles of various diameters (18–32 gauge) were evaluated in an agarose gel model to assess the flow rate at which reflux occurred. The design used in the animal studies consisted of a 27-gauge cannula needle (Terumo Medical Corp., Elkton, MD) with glued-in fused silica tubing having an outer diameter of 168 μ m and an inner diameter of 102 μ m (Polymicro Technologies, LLC, Phoenix, AZ). The needle with fused silica inside in turn was glued to tubing (Super Glue Pen; Ross, Inc., Columbus, OH) attached to the CED infusion system, as described later for the rat and nonhuman primate studies. The fused silica tubing was cut at 1 mm for the rat study and at 5 mm for the primate study, as measured from the cannula needle tip. Reflux was defined as backflow over the step created by the smaller fused silica tubing where it was affixed to the larger-diameter 27-gauge needle.

Agents Infused

Trypan blue solution (0.4%) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). All liposomes were prepared through lipid-film hydration by using HEPES-buffered saline (pH 6.5) as the hydration buffer. Each sample was hydrated in six successive cycles of freezing (–80°C) and thawing (60°C). Unilamellar liposomes were formed through extrusion by

using a 10-ml-capacity thermostated extruder (Northern Lipids, Inc., Vancouver, BC, Canada). Extrusion was performed through polycarbonate membranes by using the appropriate pore size and the number of extrusions required to reach the desired liposome size (~ 80 nm), which was determined with light scattering (Beckman Coulter, Inc., Fullerton, CA). Cholesterol was obtained from Calbiochem (San Diego, CA); the DOPC and PEG-DSPE were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All liposomes were composed of a 3:2 (molar per molar) phospholipid/cholesterol mixture. The DOPC was used as the phospholipid component, and the PEG-DSPE quantity was 10% (molar per molar) of the total phospholipid. The liposomes were labeled with membrane-bound DiIC¹⁸ fluorescence, that is, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-perchlorate (Sigma-Aldrich Corp.) having two C¹⁸ chains. The DiIC¹⁸ content was 1% (molar per molar) of total phospholipids. Other liposomes were prepared by hydrating the lipid suspension in water-soluble sulforhodamine B (20 mM; Sigma-Aldrich Corp.) and removing unencapsulated dye by using a Sephadex G75 exclusion column. For all infusion studies, liposome concentrations consisted of 2 mM phospholipid.

The Gel Model

We used 0.5% agarose gel (Life Technologies, Inc., Gaithersburg, MD) mixed with phosphate-buffered saline for all experiments. All cannulas were fixed with a holder attached to a stereotactic frame to minimize irregularities.⁵ The tip of the step-design cannula was placed at a depth of 20 to 25 mm in the gel, and CED of trypan blue was begun. The flow rate for each standard cannula and the step-design cannula was tested at least four times to assess reflux. All pictures were taken with a digital camera (Canon SD-100; Canon U.S.A., Inc., Lake Success, NY) at the end of the projected delivery time for each flow rate.

Rat Study

The protocol for this study was approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Thirty male Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) weighing 250 to 300 g were used. While in a state of deep isoflurane (Aerrane; Ohmeda PPD, Inc., Liberty Corner, NJ) anesthesia, rats were placed in a small-animal stereotactic frame (David Kopf Instruments, Tujunga, CA). A sagittal incision was made through the skin to expose the cranium, and a burr hole was made in the skull at 0.5 mm anteriorly and 3 mm laterally from the bregma by using a small dental drill. Infusions were performed at a depth of 5 mm from the brain surface by using the CED method described previously.^{2,15,19} The infusion system consisted of three components: 1) a step-design cannula; 2) an infusion line containing the infused agent; and 3) an infusion line containing olive oil. The three components were connected using flangeless fittings (Upchurch Scientific, Inc., Oak Harbor, WA), and the step-design cannula was attached to the stereotactic frame with a holder. Oil-infusion lines were prepared and 1-ml gas-tight Hamilton syringes (Hamilton Co., Reno, NV) filled with oil were attached to a rate-controllable microinfusion pump (Bioanalytical Systems, Inc., West Lafayette, IL). Ten microliters 0.4% trypan blue solution was used to determine the V_d in the rat brain. When the step-design cannula reached a depth of 5 mm, infusion was started. Flow rates of trypan blue were 0.5, 5, 10, 20, and 50 μ l/minute (four cycles for each flow rate). Ten microliters DiIC¹⁸-liposomes was infused at 0.5 and 5 μ l/minute (five cycles for each flow rate) by using the same CED technique as that used for trypan blue infusion. The infusion cannula remained in position for another 5 minutes after the delivery of trypan blue or DiIC¹⁸-labeled liposomes was finished. Rats were killed immediately after the step-design cannula was removed from the skull, and the brains were removed and frozen in dry ice-cooled isopentane. Fresh frozen brains were cut into 25- μ m-thick sections on a cryostat, and the brain tissue sections were placed on microscope slides. The V_d was analyzed using a Macintosh-based image analysis system (NIH Image, version 1.62; National Institutes of

Health, Bethesda, MD) as previously described.⁹ Hematoxylin and eosin staining (Richard-Allen Scientific, Kalamazoo, MI) of the sections was performed to evaluate tissue damage around the cannula tip.

Nonhuman Primate Study

In collaboration with Sierra Biomedical (Sparks, NV), a study was performed to track CED of rhodamine-labeled liposomes in targeted regions of the brain in nonhuman primates. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Sierra Biomedical as well as the committee at the University of California, San Francisco. Three adult male cynomolgus monkeys (*Macaca fascicularis*) weighing 2.8 to 3.5 kg were assigned to receive intracranial infusions of rhodamine-labeled liposomes into the corona radiata and brainstem through the CED technique. The monkeys were individually housed in stainless-steel cages. The infusion system used for CED was similar to the one used in the rat study. Each monkey was initially sedated with 10 mg/kg ketamine (Ketaset; Wyeth Pharmaceuticals, Collegeville, PA) administered intramuscularly; an endotracheal tube was placed and the monkey was prepared for surgery. Isoflurane (Ohmeda PPD, Inc.) was delivered at a concentration of 1 to 3% to maintain a stable level of anesthesia. Each monkey head was placed in a stereotactic frame. Vital signs were continuously monitored during surgery. A bilateral craniotomy was performed using a dental drill to expose a 3 × 2-cm area of dura mater above the target site, over the corona radiata and brainstem. Initial infusion rates were set at 0.1 μ l/minute. The step-design cannulas were fixed with holders attached to the stereotactic frame and were slowly lowered 1 cm/minute to the target site. Arachnoid layers of primate brain were traversed using the step-design cannula. An initial infusion rate of 0.1 μ l/minute was applied; this rate was increased at 10-minute intervals to a maximum of 5 μ l/minute. The total infusion volume within each target site was as follows: corona radiata 700 μ l and brainstem 700 μ l. Approximately 15 minutes after infusion, the step-design cannula assembly was slowly removed from the cortex. After completion of the neurosurgical procedure, each monkey was killed with an overdose of sodium pentobarbital, and the brain was removed and freshly frozen in dry ice-cooled isopentane. The brain tissue was later sectioned (40 μ m thick) using a cryostat and mounted onto slides, and the targeted regions were analyzed for fluorescence-generating regions by using an ultraviolet transilluminator (Bio-Rad Laboratories, Inc., Hercules, CA) to assess liposome distribution.

Results

Agarose Gel Study

First, the 18- to 32-gauge metal cannula needles were evaluated and the flow rate at which reflux occurred with each needle was assessed (Fig. 1a and b). Standard cannula needles allowed minimal distribution in agarose gel when a reflux threshold was reached (Fig. 1c). The 32-gauge cannula needle, which is one of the smallest cannula needles commercially available, demonstrated reflux at a flow rate of approximately 5 μ l/minute in agarose gel. We then turned to fused silica tubing, which offers very small diameters at an acceptable level of stability. The flow rate with the fused silica tubing was 5 μ l/minute—the rate at which infusion with the metal cannula needles started to permit reflux. Similar to the 32-gauge metal cannula needle, the fused silica tubing (outer diameter 168 μ m and inner diameter 102 μ m) also allowed reflux at a 5- μ l/minute flow rate (Fig. 1d). To create a design that might prevent reflux at the step, fused silica tubing was glued into a 27-gauge cannula needle (Fig. 1e). The tubing was cut 1 mm distal from the cannula needle tip to adjust the device to the size of the rat brain.

In the next series of experiments, reflux was assessed for the new step-design cannula combined with fused silica tubing. The flow rates used for delivery were 0.5, 5, 10, 20, and 50 $\mu\text{l}/\text{minute}$, with a total volume of 10 μl trypan blue in agarose gel. Delivery time lasted 20 minutes at a flow rate of 0.5 $\mu\text{l}/\text{minute}$, 2 minutes at 5 $\mu\text{l}/\text{minute}$, 1 minute at 10 $\mu\text{l}/\text{minute}$, 30 seconds at 20 $\mu\text{l}/\text{minute}$, and 12 seconds at 50 $\mu\text{l}/\text{minute}$. No reflux occurred with the new step-design cannula. The distribution of trypan blue was ball shaped from 0.5 to 5 $\mu\text{l}/\text{minute}$, became irregular at 10 $\mu\text{l}/\text{minute}$, and had a disk shape at 20 and 50 $\mu\text{l}/\text{minute}$ (Fig. 1f–j). Based on results of the study in agarose gel, reflux with the step-design cannula in the rats defined as the backflow of dye over the cannula step created by the smaller fused silica tubing where it was affixed to the larger-diameter 27-gauge needle.

Distribution of Trypan Blue and DiIC¹⁸-Liposomes in the Rat Brain

In the rats, flow rates and delivery times were the same as those in the agarose gel study: 20 minutes at a flow rate of 0.5 $\mu\text{l}/\text{minute}$, 2 minutes at 5 $\mu\text{l}/\text{minute}$, 1 minute at 10 $\mu\text{l}/\text{minute}$, 30 seconds at 20 $\mu\text{l}/\text{minute}$, and 12 seconds at 50 $\mu\text{l}/\text{minute}$, with a total volume of 10 μl trypan blue. Each of the 20 rats had 10 μl trypan blue infused into each hemisphere to assess the V_d and possible reflux in the brain. The new step-design cannula allowed no sign of reflux in the rat brain at any of the delivery rates (Fig. 2a–e). The V_d was almost constant between flow rates of 0.5 and 10 $\mu\text{l}/\text{minute}$ (mean 42.8 mm^3) and decreased at 20 $\mu\text{l}/\text{minute}$ (mean 32.9 mm^3) and 50 $\mu\text{l}/\text{minute}$ (mean 22.7 mm^3 ; Fig. 2f). At the end of infusion at 20 and 50 $\mu\text{l}/\text{minute}$, the step-design cannula was left in place for 5 minutes before it was withdrawn; small amounts of trypan blue were seen to follow the track left by the cannula as the device was removed. Histological examination revealed differences in the damage to the area surrounding the tip of the step-design cannula that appeared to correlate with flow rate (Fig. 2g–j): minimal damage surrounding the cannula track was found in tissue infused at flow rates of 0.5 and 5 $\mu\text{l}/\text{minute}$, whereas a much larger area of damage was observed in the brains infused at flow rates greater than 10 $\mu\text{l}/\text{minute}$. A comparison between the 27-gauge catheter and the step-design cannula was performed at a rate of 5 $\mu\text{l}/\text{minute}$. Results showed reflux-free delivery in the rodent brain with the step-design cannula as opposed to reflux in the brain with the 27-gauge catheter (Fig. 2k).

To investigate the relationship between flow rate and V_d , 10 μl DiIC¹⁸-liposomes was infused into the rat striatum (10 rats) at a low flow rate of 0.5 $\mu\text{l}/\text{minute}$ (Fig. 3a) and a high flow rate of 5 $\mu\text{l}/\text{minute}$ (Fig. 3b). Results showed a similar V_d for 0.5 $\mu\text{l}/\text{minute}$ (mean $46.3 \pm 6.6 \text{ mm}^3$) and 5 $\mu\text{l}/\text{minute}$ (mean $43.5 \pm 4.2 \text{ mm}^3$; Fig. 3c). No fluorescence was detected on the surface of the brain or along the upper part of the needle track in the histological slides, indicating a lack of reflux at both infusion rates.

Liposome Delivery in Nonhuman Primates

The dimensions of the step-design cannula were adjusted to the brain size of the cynomolgus monkeys (for use in the nonhuman primate study) by cutting the fused silica tubing 5 mm distal to the needle tip (Fig. 4a). Reflux-free delivery of 100 μl trypan blue at a 5- $\mu\text{l}/\text{minute}$ flow rate was performed in agarose gel before the animal studies to confirm the reflux-free properties of our device (Fig. 4b). Reflux-free delivery of 700 μl rhodamine-labeled liposomes into the corona radiata was achieved (Fig. 4c). Similarly, reflux-free infusion was achieved with the delivery of 700 μl rhodamine-labeled liposomes into the monkey brainstem (Fig. 4d). The maximal flow rate used for delivery to both locations was 5 $\mu\text{l}/\text{minute}$. Despite the anatomical heterogeneity of the nonhuman primate brain, our step-design cannula permitted reflux-free delivery at different depths and in different regions of brain. We use this reflux-resistant cannula in all of our current experiments.

Discussion

In the agarose gel study in which the reflux rate of cannula needles of different diameters was evaluated, we confirmed findings from previously reported studies in rodents that a smaller-diameter cannula permits a higher flow rate of CED. The 32-gauge catheter needle—the smallest-diameter needle commercially available—and even smaller-diameter fused silica tubing allowed reflux at a flow rate of approximately 5 $\mu\text{l}/\text{minute}$ in agarose gel, demonstrating the lack of rigidity for clinical application and limitations of current catheter designs for CED. In contrast, our new reflux-free step-design cannula permitted reflux-free delivery at a flow rate as high as 50 $\mu\text{l}/\text{minute}$ in agarose gel. This rate in turn enhanced CED and consequently shortened the time needed to deliver a specified volume. The step-design cannula also allowed effective reflux-free delivery of dyes and liposomes in the brains of rats and cynomolgus monkeys.

A limitation of this device for CED in rodent brains was that infusion rates of 10 $\mu\text{l}/\text{minute}$ and greater induced increasing tissue damage at the cannula tip. Trypan blue was seen to follow the track left by the cannula in the rat brain after removal of the step-design catheter at infusion rates of 20 and 50 $\mu\text{l}/\text{minute}$, strongly indicating that these high delivery speeds exceed the convective bulk flow of fluid in brain tissue and causing local compression of the brain by the infusate, which results in a decreased volume of distribution and tissue damage. Our findings indicated that the step-design of the cannula allows reflux-free delivery. Several observations support the assertion that high delivery speeds reduce V_d and cause tissue damage. We believe that the irregular and disk-shaped distribution patterns of trypan blue in agarose gel starting at a flow rate of 10 $\mu\text{l}/\text{minute}$ also reflects an increased build-up of pressure leading to the tissue damage observed in rodent brains. In a similar manner, when intracerebral hemorrhage occurs, local pressure induced by the blood leaking from the collapsed vasculature damages the surrounding brain tissue by pressing against normal brain tissue. Perhaps, irrespective of the size of the cannula, a flow rate greater than 10 $\mu\text{l}/\text{minute}$ will likely induce local tissue damage.

Data in this study demonstrated effective reflux-free delivery of liposomes as a carrier for CED-mediated drug delivery in rat and nonhuman primate brains by using the step-design cannula. The flow rate providing safe and effective delivery ($< 5 \mu\text{l}/\text{minute}$) was the same as that used in recent clinical trials. In current clinical trials, however, this flow rate required CED lasting several hours to days to deliver clinically significant volumes (36 ml) of drug to a targeted site. Positioning of catheters within the CNS (too close to the subarachnoid spaces, ventricles, or tumor cavities) has been an additional problem in clinical trials that might benefit from improved catheter design. Moreover, given the likelihood of reflux in current trials, the total volume delivered to the targeted site cannot be predicted; thus, reflux must be avoided, especially when applying CED in clinical studies involving cytotoxic agents because leakage of fluid at the surface of the brain could cause complications. Perhaps some of the side effects occurring in clinical trials involving the use of current infusion cannulas, which are not designed to prevent reflux of the infusate, can be attributed to fluid leakage at the brain surface. Given that the step-design cannula greatly minimized dye following the track made by the cannula to the surface of the agarose gel and to cortical surfaces in the experimental animals, it may provide greater safety and effectiveness in drug delivery.

We believe that reflux-free CED can reduce both the infusion time and the volume of drug required to cover the targeted structure in the brain, which in turn could shorten the duration of delivery and perhaps enhance its safety. The reflux-free step-design catheter we describe may improve the efficacy of CED in clinical trials, making this means of distribution a more reliable technique and easier to perform.

Conclusions

Improvement of current CED methods is the key to broad clinical applicability of this promising therapeutic procedure. We developed a novel step-design cannula to overcome the present limitations of CED, primarily low infusion speed and reflux of the delivered agent. The cannula was evaluated initially in agarose gel and then in rat and nonhuman primate brain. We believe that the reflux-free step-design catheter is a tool that can improve current CED protocols in terms of duration, precision, and safety, thus leading to wide clinical use of CED in the near future. Remaining to be established, however, is how tissue damage and volume of delivery are related to each other and how that relationship should be considered in therapeutic protocols. Because of the possible occurrence and consequences of reflux in current clinical trials of CED therapies, it is likely that the volume of delivery for the human brain is at present higher than would be necessary if the reflux-free step-design catheter we describe were used. Note that a revision of the volume of delivery for the new step-design catheter is currently being investigated.

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Abbreviations used in this paper

CED	convection-enhanced delivery
CNS	central nervous system
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
PEG-DSPE	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
V_d	volume of distribution

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Fig. 1.

a: Graph depicting the flow rate (microliter per minute) of reflux for each catheter needle diameter used (18-32 gauge) in agarose gel for the delivery of trypan blue. b: Photograph illustrating the millimeter scale and catheter needles used in Fig. 1a. GA = gauge. c: A 22-gauge catheter needle allowing reflux at a 0.8-μl/minute flow rate. d: Fused silica tubing allowing reflux at a 5-μl/minute flow rate. e: The step-design cannula with fused silica tubing inside cut 1 mm from the cannula tip (12.5 × 1-mm scale, no infusion performed). f: A step-design cannula allowing a 0.5-μl/minute flow rate and 10-μl delivery volume. g: A step-design cannula permitting a 5-μl/minute flow rate and 10-μl delivery volume. h: A step-design cannula allowing a 10-μl/minute flow rate and 10-μl delivery volume. i: Step-design cannula allowing a 20-μl/minute flow rate and 10-μl delivery volume. j: Step-design cannula permitting a 50-μl/minute flow rate and 10-μl delivery volume.

Fig. 2.

Histological sections of rodent brain after delivery of 10 μ l trypan blue at the following flow rates: 0.5 μ l/minute (a), 5 μ l/minute (b), 10 μ l/minute (c), 20 μ l/minute (d), and 50 μ l/minute (e). f: Bar graph showing constant V_d from 0.5- to 10- μ l/minute flow rate and decreasing V_d at 20 and 50 μ l/minute (four cycles for each flow rate). g-j: Tissue damage revealed by H & E staining at the silica tip for the 0.5-, 5-, 10-, and 50- μ l/minute flow rates. k: Tissue reflux after 20- μ l trypan blue infusion on the 27-gauge catheter side (*right*) compared with no reflux seen on the step-design cannula side (*left*).

Fig. 3. Photomicrographs of tissue sections exhibiting delivery of 10 μl DiIC¹⁸-liposomes into the rat striatum at flow rates of 0.5 $\mu\text{l}/\text{minute}$ (a) and 5 $\mu\text{l}/\text{minute}$ (b). c: Bar graph depicting the V_d for 10 μl DiIC¹⁸-liposomes at 0.5- $\mu\text{l}/\text{minute}$ (five) and 5- $\mu\text{l}/\text{minute}$ (five) flow rates. Regions generating fluorescence were delineated, and those areas were estimated by using an imaging analysis system.

Fig. 4.

a: Photograph illustrating the step-design cannula used in the monkey study. b: Image depicting the distribution of 100 μ l trypan blue in agarose gel at a 5- μ l/minute flow rate. c: Tissue section demonstrating the delivery of 700 μ l rhodamine-labeled liposomes into primate corona radiata (*arrows*). d: Tissue section revealing delivery of 700 μ l rhodamine-labeled liposomes into primate brainstem (*arrows*).