

Systemic Absorption of Ocularly Administered Enkephalinamide and Inulin in the Albino Rabbit: Extent, Pathways, and Vehicle Effects

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Abstract □ The systemic absorption of ocularly applied tritiated [D-Ala²]metenkephalinamide (YAGFM) and inulin was studied in the albino rabbit with respect to rate, extent, pathways, and vehicle effects and compared with epinephrine. Peak concentration was achieved within 20 min except for inulin, for which absorption was still ongoing at 120 min. For YAGFM, the apparent absorption rate was slower than the elimination rate, thus obeying "flip-flop" pharmacokinetics. Based on the area under the plasma concentration curve from zero to 120 min, the percent of dose systemically absorbed was 36.1 ± 4.4% for YAGFM, at least 3.3 ± 0.2% for inulin, and 58.5 ± 4.4% for epinephrine. This suggests that loss of drug to the systemic circulation is a more important factor in reducing the ocular absorption of YAGFM than for inulin. The conjunctival mucosa played as important a role as the nasal mucosa in the systemic absorption of YAGFM, while playing a secondary role in the case of inulin. Unlike nonpeptide drugs, the systemic absorption of ocularly administered YAGFM and inulin was not adversely affected by incorporation in 5% polyvinyl alcohol. Overall, the contact time of the instilled dose with the conjunctival and the nasal mucosae, their intrinsic permeability, and the extent of dilution of the instilled dose are key factors determining the vehicle effects on the extent of systemic absorption of ocularly applied peptides.

During the past decade, over ten biologically active peptides have been investigated relative to their biological effects and occurrence in the eye. These include enkephalins, atriopeptins, calcitonin gene-related peptide, vasoactive intestinal peptide, somatostatin, and substance P.¹⁻³ Although their physiological and pathophysiological significance in ocular processes such as aqueous humor dynamics and inflammation is still not understood, it is reasonable to expect that a few of these, or their analogues, will eventually become useful therapeutic agents in ophthalmology. Indeed, several peptides and proteins, notably cyclosporine,⁴ various growth factors,⁵ α -interferons,⁶ and interleukins,⁷ have already been investigated as potential therapeutic agents in uveitis, wound healing, herpes simplex infections, and induction of an immune response to rid the otherwise immune-privileged eye of an offending agent.

A factor potentially limiting the extent of ocular absorption of topically applied peptides and proteins is their loss to the systemic circulation. This could occur as a result of contact of the instilled solution with the conjunctival and the nasal mucosae, both of which have been shown to participate in the systemic absorption of ocularly applied drugs.^{8,9} The nasal mucosa, in fact, permits nearly complete absorption of drugs such as propranolol,^{10,11} progesterone,¹² testosterone,¹³ naloxone,¹⁴ nicardipine,¹⁵ and clofilium tosylate.¹⁶ Indeed, these findings are partly responsible for the recent intense interest in this route as an alternative to the oral and parenteral routes for systemic peptide and protein adminis-

tration.¹⁷ The possibility that ocularly applied peptides are absorbed systemically to elicit side effects must be addressed as a complicating factor in ocular peptide delivery.

Thus, the objectives of this study were to determine: (1) the rate and extent of systemic absorption of topically applied [D-Ala²]metenkephalinamide (YAGFM; MW 587) and inulin (MW 5000), relative to epinephrine (MW 183), in the albino rabbit; (2) the relative contribution of the conjunctival and the nasal mucosae to the systemic absorption of YAGFM and inulin; and (3) the influence of altering the relative residence time of the applied dose at the conjunctival and the nasal mucosae on the systemic absorption of YAGFM and inulin as a result of incorporation in a 5% polyvinyl alcohol solution. An analogue of methionine enkephalin, YAGFM is designed to circumvent degradation by aminopeptidases, thereby enhancing potency.¹⁸ Inulin is a linear polymer of D-glucose and D-fructose in a molar ratio of 1:20 and is metabolically stable.¹⁹ Both have been shown to permeate the cornea in small amounts following ocular administration.^{20,21}

Experimental Section

Materials—[³H]inulin (sp. act. 1.03 Ci/mmol) was purchased from Amersham (Arlington Heights, IL), and tritiated [D-Ala²]metenkephalinamide [³H]YAGFM; sp. act. 34.4 Ci/mmol) and [³H]epinephrine (sp. act. 7.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The labeled inulin was found to be homogeneous in molecular weight by gel filtration chromatography on a Sephadex G-25 column with a 0.3% NaCl solution as the mobile phase, and was used without further purification. Prior to the preparation of dosing solutions, flash evaporation was used to remove the solvents in which the labeled YAGFM and epinephrine were dissolved.

Unlabeled YAGFM, inulin, and *d,l*-epinephrine HCl were purchased from Sigma Chemicals (St. Louis, MO) and were used as received. Male albino New Zealand rabbits, weighing 2.5–3 kg, were purchased from ABC Rabbitry (Pomona, CA).

Preparation of Dosing Solutions—Dosing solutions were prepared in a phosphate buffer at pH 7.4, spiked with 0.25–0.5 mCi of labeled material per milliliter of solution to yield a final concentration of 50 μ M for YAGFM and epinephrine and 500 μ M for inulin, and rendered isotonic by adding NaCl. A more concentrated solution was used for inulin to accommodate the amount of radioactive material required. Solutions of epinephrine also contained 0.2% NaHSO₃, which was necessary to minimize epinephrine oxidation. All dosing solutions were freshly prepared immediately prior to each experiment.

Systemic Drug Bioavailability following Topical Solution Instillation—A separate group of six rabbits was used for YAGFM, inulin, and epinephrine. Prior to dosing, each rabbit was cannulated in a central ear artery, using polyethylene tubing (PE-50, Intramedic), and heparinized with 500 units of Na heparin (Western Medical Supply, Arcadia, CA). Within the next 15 min, 25 μ L of a dosing

solution was instilled onto each eye of the rabbit. Two-milliliter blood samples were collected into heparinized tubes at 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min and stored on ice. The volume of blood aspirated was replenished with an equal volume of dextrose.

The blood samples were centrifuged at $1000 \times g$ for 5 min in a refrigerated high-speed centrifuge (Sorvall model RC-5B, DuPont Instruments, Newton, CT). One milliliter of the resultant plasma containing inulin or epinephrine was transferred to a glass scintillation vial containing 12 mL of a scintillation cocktail (Instagel, Packard, Downers Grove, IL) and refrigerated for at least 12 h before counting in a liquid scintillation spectrometer (Beckman LSC-7500, Irvine, CA). No attempts were made to distinguish epinephrine from its metabolites. After correcting for quenching, the data in counts per minute were converted to pmoles of drug using the external standard method. The level of radioactivity was not considered to be significant unless the counts were greater than two standard deviations above the mean of control blank samples. It was predetermined that no tritium exchange occurred over the time course of the experiment and on storage.

Plasma containing YAGFM was precipitated by an equal volume of 0.3 M perchloric acid and centrifuged. The pellet was found to contain <5% of the radioactivity in the plasma sample. Intact YAGFM in the supernatant was measured from the amount of radioactivity adsorbed onto polystyrene beads, as previously described.²¹ Metabolized YAGFM was measured from the radioactivity remaining in the supernatant. The metabolite was most probably Tyr-D-Ala-Gly (YAG) as a result of the action of vascular endothelial-associated angiotensin converting enzyme on the peptide.^{22,23}

The systemic bioavailability of a given topically applied compound was determined by comparing its area under the concentration-time curve (AUC) from zero to 120 min to that obtained in a new group of rabbits to whom an intravenous bolus was administered. The AUC was determined using the trapezoidal rule.

Pathways of Systemic YAGFM and Inulin Absorption following Topical Solution Instillation—It was assumed that the conjunctival and the nasal mucosae both participated in the systemic absorption of topically applied drugs. Possible loss of drug to the gastrointestinal tract, where systemic absorption may also occur,²⁴ was considered as part of nasal drug absorption. To determine the involvement of the conjunctival mucosa in the systemic drug absorption, 25 μL of a 50 μM YAGFM or a 500 μM inulin solution was instilled in the conjunctival sac of each eye of six rabbits. The conjunctival sac was isolated from the nasolacrimal duct by a punctum plug, which was fashioned from a 5-mm segment of a polyethylene tubing (PE 50, 1 mm o.d.) that had been heat sealed at one end and beveled at the other.²⁵ To determine the contribution of the nasal mucosa to systemic drug absorption, 25 μL of a 50 μM YAGFM or 500 μM inulin solution was instilled nasally via a polyethylene catheter (PE 50, 1 mm o.d.) preinserted 15–20 mm into each lacrimal sac/nasolacrimal duct of another group of rabbits. In each instance, serial blood samples were collected from each rabbit up until 120 min and

assayed for drug as described earlier. The fraction of dose systemically absorbed from each mode of administration was determined as described earlier and compared with one another using one-way analysis of variance and Tukey's multiple comparison test.²⁶

The following terminology will be used to denote the various modes of administration: "ocular" for instillation in rabbits with open nasolacrimal ducts (these comprised the control group); "conjunctival" for instillation in rabbits with closed nasolacrimal ducts; and "nasal" for instillation directly in the nasolacrimal duct.

Effect of 5% Polyvinyl Alcohol (PVA) Solution on the Systemic Absorption of Topically Applied YAGFM and Inulin—This was determined by instilling 25 μL of a 50 μM YAGFM or a 500 μM inulin solution in 5% PVA to each eye of six rabbits. Blood samples were collected and processed as described earlier. The viscosity of PVA, as determined using a cone and plate viscometer (Brookfield Digital Viscometer model LVTDCP-600, Stoughton, MA) at a shear rate of three (spindle #18) and a temperature of 34 $^{\circ}\text{C}$, was ~ 70 cps.

Results

The time course of drug concentration in the plasma following topical solution instillation of YAGFM, inulin, and epinephrine, in comparison with an intravenous bolus, is shown in Figure 1. Peak concentration was achieved within 20 min for YAGFM and epinephrine. However, systemic inulin absorption was still ongoing at 120 min. Based on the AUC, the systemic bioavailability of YAGFM, inulin, and epinephrine was $36.1 \pm 4.4\%$, at least $3.3 \pm 0.2\%$, and $58.5 \pm 4.4\%$, respectively. The systemic bioavailability for inulin was probably an underestimate since absorption was still ongoing at the last sampling time point. Consequently, the comparison of this value to other data must be viewed in this light.

The slope of the terminal phase following topical administration of YAGFM was shallower than that following intravenous administration. This is indicative of "flip-flop" pharmacokinetics, which has also been observed following the nasal administration of metkephamid,²⁷ another metabolically stable methionine enkephalin analogue.²⁸ Simultaneous nonlinear regression analysis of the pooled data obtained from these two routes to a one-compartment model yielded an apparent absorption rate constant of $0.017 \pm 0.007 \text{ min}^{-1}$ and an apparent elimination rate constant of $0.097 \pm 0.004 \text{ min}^{-1}$. In comparison, the respective rate constants for epinephrine were 0.19 ± 0.05 and $0.0095 \pm 0.0008 \text{ min}^{-1}$. Nonlinear iterative curve fitting was done on an IBM PC-XT using the statistical package PCNONLIN (Statistical Consultants, Inc., Lexington, KY). The data

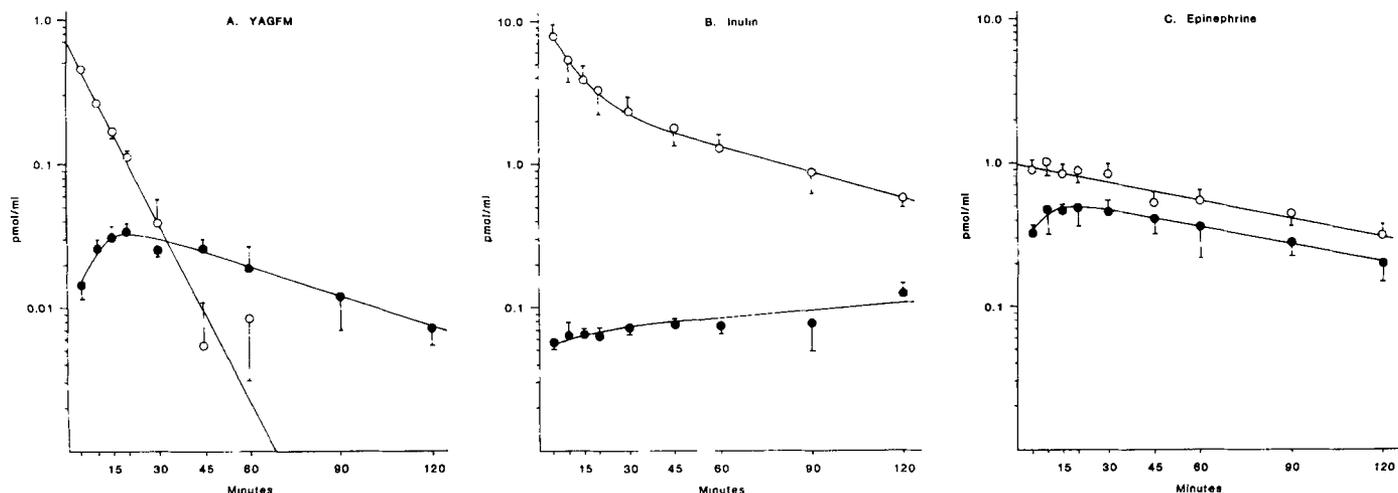


Figure 1—Plasma concentration-time profiles of intact YAGFM (A), inulin (B), and epinephrine (C) following intravenous (O) and ocular (●) administration. The lines for YAGFM and epinephrine were drawn by

nonlinear regression analysis as described in the text. Those for inulin were drawn by visual inspection. Error bars represent standard error of the mean, and six rabbits were used per compound.

points were weighted by the inverse of their respective variances.

The metabolism of YAGFM following both intravenous and ocular administration (with open duct) was rapid (Figure 2). Under both conditions, about half of the radioactivity detected in the plasma at 5 min was in the form of metabolite(s). However, following ocular administration, a smaller proportion of the radioactivity between 20 and 60 min existed as metabolites.

The concentration-time profiles of YAGFM and inulin following direct nasal administration, conjunctival administration, ocular administration in 5% PVA, and ocular administration in buffer are displayed in Figure 3, and the corresponding apparent absorption and elimination rate constants for YAGFM are shown in Table I. One way analysis of variance revealed no difference in the apparent elimination rate constants. However, the apparent absorption rate constant was similar for nasal administration and ocular administration in buffer, whereas it was similar for conjunctival administration and ocular administration in 5% PVA.

The percent of YAGFM and inulin absorbed under each of the aforementioned conditions is shown in Table II. For YAGFM, occluding the nasolacrimal duct and instilling the solution nasally both resulted in a 50% reduction in systemic bioavailability when compared with the control ($p < 0.05$), whereas ocular administration in 5% PVA resulted in a 1.4-fold increase in systemic bioavailability ($p < 0.05$). For inulin, neither occluding the nasolacrimal duct nor ocular administration in 5% PVA improved the systemic bioavailability ($p = 0.05$), although instilling the solution nasally improved systemic bioavailability twofold ($p < 0.05$).

The metabolism of YAGFM following nasal administration at 5 and 10 min was less extensive when compared with conjunctival administration and ocular administration in 5% PVA (Figure 2). Moreover, it was less extensive over half of the time course when compared with the control (i.e., ocular administration in the presence of an open nasolacrimal duct; Figure 2).

Discussion

This study demonstrates that the extent of systemic absorption of ocularly administered YAGFM is similar to that of epinephrine and is ~10 times greater than that of inulin. Systemic drug loss is therefore a more important factor limiting the ocular absorption of YAGFM than for inulin. Inulin further differs from YAGFM in that its systemic absorption appears to be sustained (Figure 1). This suggests that inulin may be trapped in the mucus layer or the glycocalyx on the conjunctival and nasal surfaces, resulting in retardation of its entry into the systemic circulation.^{29,30} This is a distinct possibility in light of the recent finding of Wiedmann and Robinson³¹ that bovine submaxillary mucin, when applied to conjunctival cell monolayers, increased the overall resistance to the penetration of fluorescein esters.

Table I—Apparent Absorption (k_a) and Elimination (k_e) Rate Constants following Ocular and Nasal Administration of YAGFM in the Albino Rabbit^a

Condition	k_a, min^{-1}	k_e, min^{-1}
Ocular administration		
Open nasolacrimal duct	0.017 ± 0.007	0.097 ± 0.004
Closed nasolacrimal duct	0.041 ± 0.010	0.098 ± 0.004
5% Polyvinyl alcohol	0.030 ± 0.003	0.097 ± 0.004
Nasal administration	0.017 ± 0.004	0.097 ± 0.004

^a Values are expressed as mean \pm SEM; $n = 6$; dose was 25 μL of 50 μM YAGFM.

Table II—Percent of Dose Absorbed following Ocular and Nasal Administration of YAGFM and Inulin in the Albino Rabbit^a

Condition	YAGFM ^b	Inulin ^c
Ocular administration		
Open nasolacrimal duct	36.1 ± 4.4	3.3 ± 0.2
Closed nasolacrimal duct	16.7 ± 2.5^d	3.2 ± 0.7
5% Polyvinyl alcohol	50.7 ± 3.2^d	3.6 ± 0.7
Nasal administration	17.6 ± 3.0^d	6.5 ± 1.1^d

^a Values are expressed as mean \pm SEM, $n = 6$. ^b 25 μL of 50 μM YAGFM. ^c 25 μL of 500 μM inulin. ^d Significantly different from the respective control (open nasolacrimal duct) at $p < 0.05$ using Tukey's t -test for multiple comparisons.

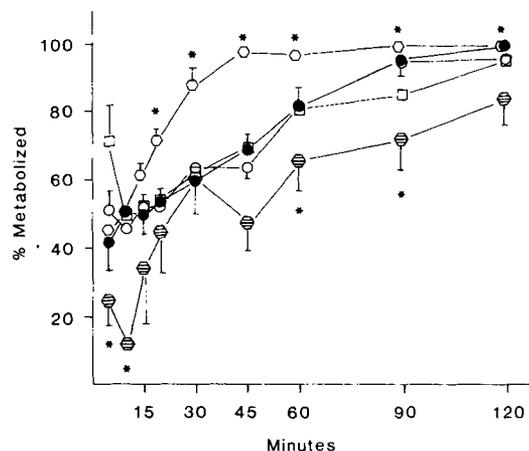


Figure 2—Time course of percent of total radioactivity (mean \pm SEM) attributed to metabolite following intravenous, nasal, and ocular administration of 2.5 μmol of YAGFM in the albino rabbit. Asterisks denote significant difference from ocular administration at $p < 0.05$. Error bars represent standard error of the mean; where not shown, they are smaller than the size of the symbol. Six rabbits were used per condition. Key: (\circ) intravenous; (\square) ocular; (\odot) nasal; (\bullet) conjunctival; (\square) 5% polyvinyl alcohol.

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The low systemic bioavailability of ocularly applied inulin is consistent with its molecular size.³² The reduced bioavailability of YAGFM compared with epinephrine is consistent with its 10 times smaller absorption rate constant and, to some extent, its 10 times larger elimination rate constant. The rapid systemic clearance of many peptides is well documented,³³ whereas the difficulty experienced by peptides in traversing various epithelia is to be expected from their physicochemical properties, notably hydrophilicity and molecular size.³²

Ocularly administered YAGFM and inulin are probably absorbed into the systemic circulation via the blood vessels in both the conjunctival and the nasal mucosae. Based on the

concentration–time profiles obtained from conjunctival and nasal administration in comparison with that obtained following ocular administration (Figure 3), the conjunctival mucosa appears to play as important a role as the nasal mucosa in contributing to the systemic absorption of YAGFM. This is unlike timolol, for which the conjunctiva plays a secondary role.⁹ It is therefore not surprising that slowing the appearance of the instilled solution in the nasal passages through the use of 5% PVA did not reduce the systemic bioavailability of YAGFM (Figure 3, Table II), as it did for timolol.³⁴ Consequently, the improved absorption of YAGFM afforded by 5% PVA over nasal administration in buffer is likely due to reduced ciliary clearance^{35,36} of the ocularly applied peptide reaching the nasal cavity. This is

consistent with the findings of Morimoto et al.³⁷ that 0.1 and 1% polyacrylic acid gel enhanced the nasal absorption of insulin and [Asu^{1,7}]-eel calcitonin in the rat.

The incorporation of inulin in 5% PVA resulted in a concentration–time profile distinctly different from those derived from the other modes of administration (Figure 3). Absorption was delayed, suggesting that the conjunctival mucosa was not as permeable to inulin as was the nasal mucosa. In this light, the similarities in the concentration–time profiles and the percent of dose absorbed between conjunctival and ocular administration (Figure 3, Table II) was probably due to the prolonged contact of inulin with the conjunctival mucosa afforded by 5% PVA. The improved absorption of inulin when administered nasally, on the other hand, was most probably due to lesser dilution of the applied dose, resulting in a higher drug concentration in the nasal passages and, therefore, a steeper concentration gradient for drug penetration. Collectively, these findings suggest that the contact time of the instilled dose with the conjunctival and the nasal mucosae, their intrinsic permeability, and the extent of dilution of the instilled dose are key factors determining the vehicle effects on the extent of systemic absorption of ocularly applied peptides.

In addition to the permeation barrier, metabolism of YAGFM during permeation across the conjunctival and the nasal mucosae is another barrier limiting its systemic bioavailability. Previous work in this laboratory revealed that both tissues possess proteases capable of metabolizing YAGFM.^{38,39} The rate of degradation was faster in the nasal than in the conjunctival homogenate. Yet the percentage of radioactivity in plasma appearing as metabolite was less when dosing was limited to the nasal as compared with the conjunctival mucosa (Figure 2). One interpretation of this finding is that the subcellular compartmentalization of the proteases and therefore the probability of their contact with YAGFM during absorption are different in these two mucosae. Another interpretation is that YAGFM was more rapidly absorbed across the conjunctival than the nasal mucosa into the systemic circulation, where the bulk of YAGFM was metabolized. Indeed, the difference in metabolic clearance during passage across the conjunctival and the nasal mucosae must not be great enough to yield different YAGFM systemic bioavailability for the two routes of administration (Figure 3, Table II).

In summary, YAGFM and inulin are absorbed into the bloodstream following topical ocular administration, the former being more efficiently absorbed than the latter. It is anticipated that depending on their molecular size, lipophilicity, and susceptibility to proteolysis both preceding and during penetration, varying extents of other peptides and proteins can also be absorbed. The conjunctival mucosa appears to play as important a role as the nasal mucosa in the systemic absorption of ocularly applied YAGFM, while playing a secondary role in the case of inulin. This is evident in the effect of 5% PVA on the rate and extent of systemic absorption of these two substances.

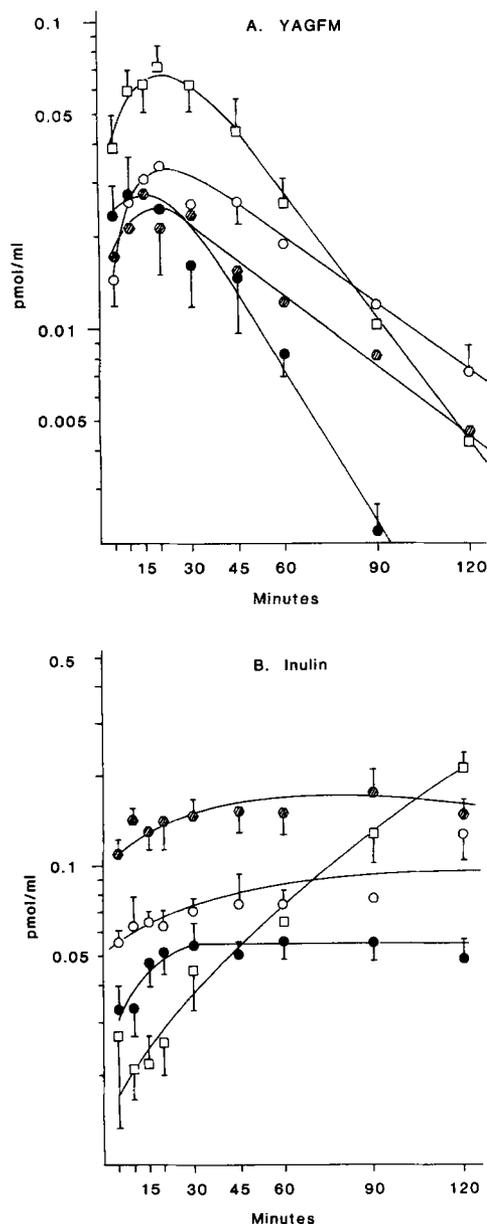


Figure 3—Plasma concentration–time profiles of intact YAGFM (A) and inulin (B) following direct nasal administration (●), ocular administration of drug solutions in 5% polyvinyl alcohol (□), and ocular administration of drug solutions in buffer under both open (○) and occluded (●) nasolacrimal duct conditions. The lines for YAGFM were drawn by nonlinear regression analysis as described in the text. Those for inulin were drawn by visual inspection.

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