Transdermal Iontophoretic Delivery of Luteinizing Hormone Releasing Hormone (LHRH): Effect of Repeated Administration

Mark C. Heit,[1] Nancy A. Monteiro-Riviere,[1] Friederike L. Jayes,[2] and Jim E. Riviere[1,3]

Received April 1, 1993; accepted February 8, 1994

The transdermal iontophoretic delivery of the reproductive peptide hormone, luteinizing hormone releasing hormone (LHRH) is investigated in the isolated perfused porcine skin flap model (IPPSF). LHRH is delivered twice in a single flap experiment in efforts to identify factors inherent to iontophoretic delivery that might effect the drug flux of a subsequent iontophoretic episode. Initial iontophoretic delivery of LHRH is quite reproducible; however, subsequent iontophoretic episodes result in widely divergent fluxes thought to be caused by iontophoretic influences on the skin. Iontophoretic application of a drug on a previous active site, enhances the flux during the second application. A mass balance study is performed to explain these findings. By iontophoretically delivering I^{125} labelled LHRH in the isolated perfused porcine skin flap model, the entire iontophoretic dose is identified and quantified. A drug depot is identified in the skin underlying the electrode which is approximately two times as large as the entire mass of drug delivered systemically.

KEY WORDS: iontophoresis; peptide; LHRH; transdermal delivery.

INTRODUCTION

Peptide drugs, which are not orally active and cannot be percutaneously delivered passively, may be ideally suited to iontophoretic delivery. Peptides have been iontophoretically delivered both *in vitro* (1–3) and *in vivo* (4–7). The goals for iontophoresis vary depending on whether it is used as a systemic or topical delivery system. As the former, the achievement of reproducible therapeutic blood concentrations is critical; however, used topically, the goal is to confine drug to the skin. The fate of drug once it has entered the skin is largely unknown. The actual physiochemical mechanism of iontophoresis remains unclear and the route by which compounds pass through the skin under iontophoretic influence is currently under investigation (8). In addition, it is unclear how iontophoretic current effects skin biology, physiology, and metabolism. Before iontophoresis can be regarded as a routine alternative drug delivery system, many of these unknowns need to be addressed.

Previous studies in our laboratory demonstrated the ability to deliver luteinizing hormone releasing hormone (LHRH) by iontophoresis in the isolated perfused porcine skin flap (IPPSF) and to predict *in vivo* fluxes in pigs (9). The current study further investigates the anodal iontophoretic delivery of LHRH in the IPPSF by specifically addressing potential skin and flux effects of a second iontophoretic dosing, an area that has received scant attention. This study also addresses the fate of a compound once it has entered the skin. By iontophoretically delivering I^{125} labelled LHRH in the IPPSF model, the fate of the entire iontophoretic dose can be accounted for via mass balance.

MATERIALS AND METHODS

The IPPSF model has been described in detail elsewhere (10,11). The active and indifferent electrodes were circular 1 cm^2 (skin contact area), Porex® silver screen sandwiches. Isoelectric focussing and capillary zone electrophoresis studies performed in our laboratory and that of Becton Dickinson Research Center suggest LHRH (1182.3 MW) is highly positively charged and electrophoretically mobile at physiological pH (12). In non-radiolabelled protocols, a 1 mg/ml LHRH solution in 154 mM NaCl and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) was placed in the active positive electrode. LHRH-I^{125} (specific activity approximately 8 pg/60000 cpm) was synthesized using an iodogen reaction (13) and fractions elluted with 50 mM TRIS buffer. The dosing solution for radioactive flaps contained 10% undiluted LHRH-I^{125} and 90% nonradiolabelled LHRH dosing solution (1.127 mg/ml LHRH in 154 mM NaCl 10 mM MES) with the pH of the final solution adjusted to 6.0 with 0.25 N HCl. Due to the high specific activity of the radioactive solution, a negligible amount of LHRH-I^{125} was added resulting in a LHRH concentration of 1 mg/ml in the final dosing solution. The indifferent electrode in all experiments contained a 50% saturated NaCl/water solution (15.9 g/100 ml).

Each electrode fit snugly into polyethylene rings (12 mm ID, 23 mm OD, 1/16″) that were placed on the skin's surface such that the solution contained could not contaminate other areas. A polyvinylchloride foam circular adhesive was applied to the top of the ring and electrode to prevent evaporative loss from the electrode. In all experiments, a portable power supply (WPI stimulus isolator model A360, New Haven, Ct) was used to deliver a constant current of 0.2 mA (0.2 mA/cm^2 current density).

Repeated Iontophoresis

After a one hour acclimation period, each of the harvested skin flaps received one of two protocols designed to investigate skin site effects on iontophoretic delivery. In each protocol a 3 h 0.2 mA/cm^2 direct current was applied followed by a 2 h passive period. A new electrode is then used to apply a 2 h 0.2 mA/cm^2 second iontophoretic period followed by a 1 h washout during which there was no current. In one protocol, the new electrode used during the second iontophoretic episode was placed on the same skin site used in the first iontophoretic period. In the paired flap, a new electrode was moved to a new area of skin for the second period. In both protocols, a third ring and cover con-

[1] Cutaneous Pharmacology and Toxicology Center, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, 27606 telephone: 919-829-4305.

[2] Department of Animal Science, North Carolina State University, Raleigh, North Carolina 27606.

[3] To whom correspondence should be addressed.

0724-8741/94/0700-1000$07.00/0

taining an unstimulated dry electrode was applied to the flap for control measures. This insured that all skin sites were equally hydrated (due to occlusion of the skin) and physically manipulated prior to the second active period.

Radiolabelled Flaps

Radiolabelled flaps received a 3 h, 0.2 mA/cm^2 active period followed by a 2 h passive period during which there was no current. At this point, the experiment was terminated and the entire flap and electrode assembly analyzed for LHRH-I^{125}. Prior to skin analysis, the flap's surface was cleaned of any radioactivity (swab) and the stratum corneum removed by 12 tape strippings (strip) (Scotch 3M Magic Tape). The skin directly beneath the positive electrode was removed, embedded, and quenched in liquid nitrogen for serial sectioning as previously described (14). The entire flap was then frozen in liquid nitrogen and completely dissected for LHRH analysis.

Saline Flaps

The initial electrode in each saline flap experiment was dosed with non-radioactive LHRH solution. A 3 h 0.2 mA/cm^2 current followed by a 2 h passive period (no current) was applied. The active electrode was then replaced by a new electrode which contained the saline vehicle. The current was reapplied for one hour followed by a second passive period.

Venous perfusate samples were collected half-hourly throughout all experiments except during the second active period of saline flaps during which samples were taken every 6 min for the first half hour. Collected samples were immediately placed at −20°C until assayed. Voltage, media flow and perfusion pressure were recorded. At the termination of nonradiolabelled experiments, skin samples were taken from beneath each electrode and processed for light microscopy as previously described (15).

LHRH Radioimmunoassay

LHRH was quantified in IPPSF perfusate using a single antibody ethanol extraction radioimmunoassay (9). All samples were run in triplicate. Assay sensitivity ranged between 0.6 to 1 pg/tube at 90% binding (B/Bo). Intra- and interassay coefficient of variation were 8.2 and 16.5% at 48 pg/tube and 10.9 and 8.3% at 10.6 pg/tube.

Statistics

Areas under the flux curves were calculated using SAS (Statistical Analysis System, Cary, NC) via trapezoidal summation and were compared using a Student's *t* test. The first active periods of 23 experiments in which a new electrode was used to deliver a 3 h 0.2 mA/cm^2 current have been grouped and the flux data pooled since identical conditions were used. Comparisons made to the first active period are compared to these pooled data.

RESULTS

Previous studies performed in our laboratory (9) have shown that no LHRH enters the perfusate under passive conditions at the level of detection of the assay used. In contrast, LHRH flux was shown to rise rapidly after current was applied and quickly fall to baseline following removal of current.

The mean ± one standard error LHRH flux profile for 23 flaps in which the first 5 h of the experimental protocol is identical to the protocols in this study is shown by a solid bold line in Figure 1. The average maximum flux is 8.6 ng/min/cm^2 and an average of 1.1 μg ± 0.7 μg (corresponding to 0.7% of the dose) is delivered over the 5 h active period. There is little variation in the flux profile for this period.

Utilization of an area of the flap that was dosed previously with an active electrode allowed for higher fluxes during the second active period (Figure 1, dashed line). Similar amounts of drug were delivered in both active periods of the protocol in which a naive skin site was used for each (Figure 1, dotted line). In contrast, the amount delivered in the active period utilizing a previously used skin site, was significantly greater than that delivered in the first 2 h of the first active period ($p = .06$).

The amount and location of LHRH recovered after iontophoretic delivery of radiolabelled compound is demonstrated in Table I. On average, greater than 92% of the dose could be accounted for with the majority of that amount never entering the skin (in the electrode, swab or ring). Approximately 1.4% of the dose remained in the skin (stratum corneum + underlying skin and subcutaneous tissue (SQ) + surrounding skin) even after a 2 h washout period.

The venous perfusate flux profile for two saline flaps is shown in Figure 2. LHRH flux begins to increase shortly after the current is applied and rapidly falls to pretreatment levels. The application of a second electrode containing no LHRH causes a small iontophoretic flux which appears to peak (~1 ng/min/cm^2) at 0.5 h after current is reapplied.

Mild histological alterations resembling those previously described (15) were seen in a small percentage (<15%) of active sites. These changes did not correlate to any experimental protocol and therefore are considered random.

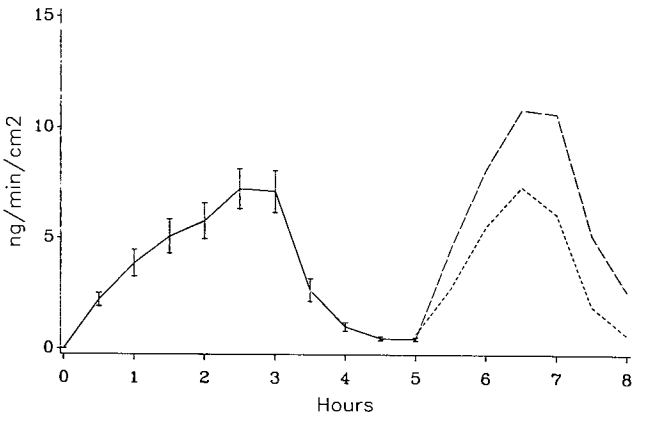

Figure 1. Transdermal iontophoretic flux of LHRH in the IPPSF illustrating the effect of delivery at a previously used skin site. The mean flux ± one standard error for 23 flaps in which experimental conditions were identical over the first 5 h are grouped and represented by a solid bold line. Significantly more LHRH is delivered in the dashed protocol between 5-7 h than in the first 2 h of the initial iontophoretic period ($p = 0.06$).

Table I. Mass balance tabulation of radiolabelled flaps (mean and range of 4 flaps).

Location	Percent of dose
positive electrode	74.25 (62–82)
negative electrode	0
ring	15.5 (11–22)
electrode assembly	89.75 (84–93)
swab	1.1 (0.4–1.7)
total not in skin	90.85 (85.7–94.2)
stratum corneum (strip)	0.73 (0.2–1.5)
underlying skin	0.35 (0.3–0.4)
surrounding skin	0.25 (0.1–0.4)
underlying SQ	0.04 (0.04)
total in skin	1.37 (0.7–2.3)
total recovery	92.2 (86.9–95.2)

DISCUSSION

It is generally thought that the stratum corneum provides the greatest barrier to transdermal delivery, and that once these layers are penetrated, compounds move easily into the dermal circulation. Once a drug has entered the epidermis and dermis, it is subject to tissue binding and metabolism. At this point, it is unclear whether passive diffusion or electrophoretic movement is responsible for delivering drug to the vasculature.

Repeated identical iontophoretic conditions should result in similar drug flux profiles, thus as a control, a new electrode was placed on a naive skin site in the first and second active periods of one protocol (Figure 1, dotted line). In this instance, similar amounts of drug were delivered in the first 2 h of the first active period as during the second active period confirming the viability and barrier integrity of the IPPSF over the entire 8 h experimental protocol.

Using an area of the skin previously used as an active site generates more flux during a second active period than when that electrode is placed at an unused portion of skin (Figure 1, dashed line). This enhanced delivery suggests that a previous iontophoretic period in some way alters the underlying skin. This alteration may involve the filling of binding sites in the skin by the first iontophoretic period such that drug delivered during the second active period is free to pass through the skin into the vasculature. Alternatively, enhanced flux may be the result of mobilization of LHRH which, during the passive period remains in the skin. When a new electrode is placed and current is reapplied drug may be delivered not only from the electrode, but also from tissue sites that have been "loaded" by the first iontophoretic period. Enhanced flux is not seen at a previously used active site however, unless the electrode-skin contact has been disrupted (e.g. lifted and replaced) (unpublished results).

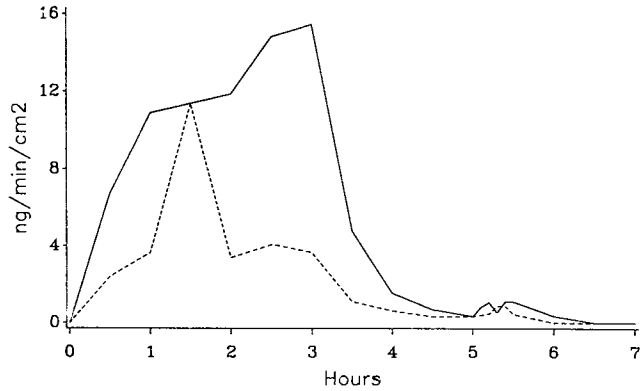

Figure 2. Normalized LHRH iontophoretic flux in two flaps in which a 0.2 mA/cm^2 direct current was applied from 0–3 h and 5–6 h. An electrode containing saline was used during the second iontophoretic period (5–6 h) (solid lines). The dosing solution was a nonradiolabelled 1.0 mg/ml LHRH solution in a buffered saline vehicle (see text for composition).

A third explanation for enhanced flux of a second iontophoretic period could be alteration in the barrier function of the skin caused by a first iontophoretic episode. Green et al. have shown iontophoresis pretreatment in some way increases the skin's permeability to subsequent passive delivery of a tripeptide (16). Similarly, iontophoretic pretreatment in this study may have decreased the skin's barrier to subsequent iontophoretic flux or may have allowed a greater contribution of passive flux to total flux. No consistent alterations in morphology, including those previously described (15), were observed to suggest increased permeability and the considerably larger molecular size of LHRH as compared to a tripeptide may have precluded any passive delivery.

Since iontophoretic delivery is theoretically inversely related to a compound's molecular weight, the LHRH-I^{125} concentrations and mass measured within the skin and perfusate in the current study may underestimate those achieved by the delivery of the native compound. Previous high performance liquid chromatographic analysis of nonradioactive perfusate samples suggest that at least 90% of the molecules remain intact (unpublished observations in our laboratory) during iontophoretic transdermal delivery, thus gamma counting of skin samples should adequately reflect intact LHRH molecules.

A reservoir effect has been demonstrated for a number of compounds (5,17). This study clearly demonstrates the presence of an LHRH depot within the skin underlying and surrounding the positive electrode corresponding to at least 1.4% of the dose. In contrast, under the experimental conditions of our study, greater than 90% of a dose on average never entered the skin while only 0.7% of the dose was delivered systemically after a 5 h active period. Although alterations in the electrode design used may allow for greater efficiency or absolute flux, the ability to manipulate drug once it has entered the skin also provides a method for affecting drug flux. If the formation of the depot identified in this study could be prevented or mobilized once formed, total drug flux could potentially double. Conversely, if in some way the drug became unable to access the vasculature or if the depot could be expanded, higher local skin concentrations could be achieved.

In an attempt to determine whether passive diffusion or electrophoretic force is responsible for drug movements within the skin and whether the delivery of "loaded" drug is responsible for enhanced flux of repeated iontophoretic episodes, a saline electrode was used to apply a current at a previously used active site at a time when drug flux was known to be negligible. A small LHRH flux was induced

(Figure 2 (5–6 h)) indicating the ability to "force" drug into the vasculature. However, a comparison of the small amount delivered (~40 ng) to that amount in the skin at that time (1.4% of a 161 μg dose or 2250 ng) suggests that greater than 98% of compound in the skin was unavailable to be delivered by this saline iontophoretic episode. The exact mechanism of this unavailability requires further investigation.

The small amount delivered during the second active period of these saline flaps is insufficient to account for the increased amount of LHRH delivered in the second iontophoretic episode of protocol 5. This suggests that a prior iontophoretic episode not only delivers a small amount of initially "loaded" drug but also facilitates the movement of drug from electrode to blood supply. Thus, if sites for drug sequestration are saturated by prior drug delivery, drug molecules being delivered from a second electrode are free to pass through the skin to access subepidermal blood vessels resulting in a greater flux. Additionally, alterations in barrier function caused by prior iontophoresis may allow for greater flux. A decrease in available binding sites would be indistinguishable from a decreased skin barrier in the present experimental design.

In summary, the significant findings of the present study include the ability to transdermally deliver LHRH, a decapeptide hormone, only under iontophoretic conditions, the enhanced iontophoretic delivery following "iontophoretic pretreatment", and the identification of a skin depot of this peptide, a small portion of which can be delivered with a saline electrode.

ACKNOWLEDGMENTS

Financial support was provided by the Becton Dickinson Research Center (BDRC), Research Triangle Park, N.C. We thank Dr. Randy Bock and Mr. Burt Sage of BDRC. We also thank Dr. Jack Britt and the NCSU Department of Animal Science for their expertise and use of facilities for the hormone assays. We acknowledge Dr. GD Niswender for supplying antisera to luteinizing hormone and we thank the technical staff at the Cutaneous Pharmacology and Toxicology Center at North Carolina State University. This work was completed in partial fulfillment of a Ph.D. at NCSU (MCH).

REFERENCES

1. R. R. Burnette and D. Marrero. Comparison between the iontophoretic and passive transport of thyrotropin releasing hormone across excised nude mouse skin. *J. Pharm. Sci.* 75(8): 738–743 (1986).
2. Y. W. Chien, O. Siddiqui, W. M. Shi, P. Lelawongs, and J. C. Liu. Direct current iontophoretic transdermal delivery of peptide and protein drugs. *J. Pharm. Sci.* 78(5):376–383 (1989).
3. V. Srinivasan, W. I. Higuchi, S. M. Sims, A. H. Ghanem, and C. R. Behl. Transdermal iontophoretic drug delivery: mechanistic analysis and application to polypeptide delivery. *J. Pharm. Sci.* 78(5):370–375 (1989).
4. R. L. Stephen, J. Petelenz, and S. C. Jacobsen. Potential novel methods for insulin administration: I. Iontophoresis. *Biomed. Biochem. Acta* 43(5):553–558 (1984).
5. O. Siddiqui, Y. Sun, J. C. Liu, and Y. W. Chien. Facilitated transport of insulin. *J. Pharm. Sci.* 76(4):341–345 (1987).
6. Y. W. Chien, O. Siddiqui, Y. Sun, W. M. Shi, and J. C Liu. Transdermal iontophoretic delivery of therapeutic peptides/proteins I: insulin. *Ann N.Y. Acad. Sci.* 507:32–50 (1987).
7. J. C. Liu, Y. Sun, O. Siddiqui, Y. W. Chien, W. M. Shi, and J. Li. Blood glucose control in diabetic rats by transdermal iontophoretic delivery of insulin. *Int. J. Pharm.* 44:197–204 (1988).
8. C. Cullander. What are the pathways of iontophoretic current flow through mammalian skin? *Adv. Drug. Del. Rev.* 9:119–135 (1992).
9. M. H. Heit, P. L. Williams, F. L. Jayes, S. K. Chang, and J. E. Riviere. Transdermal Iontophoretic Peptide Delivery: *In vitro* and *In vivo* Studies with Luteinizing Hormone Releasing Hormone (LHRH). *J. Pharm. Sci.* 82:240–243 (1993).
10. J. E. Riviere, K. F. Bowman, N. A. Monteiro-Riviere, L. P. Dix, and M. P. Carver. The isolated perfused porcine skin flap (IPPSF) 1. A novel *in vitro* model for percutaneous absorbtion and cutaneous toxicologic studies. *Fundam. and Appl. Toxicol.* 7:444–453 (1986).
11. J. E. Riviere, B. Sage, and N. A. Monteiro-Riviere. Transdermal lidocaine iontophoresis in isolated perfused porcine skin. *J. Toxicol.- Cut. & Ocular Toxicol.* 8(4):493–504 (1989/90).
12. M. C. Heit, A. McFarland, R. Bock, and J. E. Riviere. Isoelectric focussing and capillary zone electrophoretic studies using luteinizing hormone releasing hormone (LHRH) and its analog. *J. Pharm. Sci.* 83(5):654–656 (1994).
13. P. J. Fraker and J. C. Speck. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Comm.* 80(4):849–857 (1978).
14. J. E. Riviere, N. A. Monteiro-Riviere and A. O. Inman. Determination of lidocaine concentrations in skin after transdermal iontophoresis: effects of vasoactive drugs. *Pharm. Res.* 9:211–214 (1992).
15. N. A. Monteiro-Riviere. Altered epidermal morphology secondary to lidocaine iontophoresis: *In vivo* and *in vitro* studies in porcine skin. *Fundam. Appl. Toxicol.* 15:174–185 (1990).
16. P. Green, S. Braham, B. Françoise, Q. R. Pilgrim, and R. H. Guy. *In vitro* and *in vivo* iontophoresis of a tripeptide across nude rat skin. *J. Contr. Rel.* 20:209–218 (1992).
17. L. L. Wearley, K. Tojo, and Y. W. Chien. A numerical approach to study the effect of binding on the iontophoretic transport of a series of amino acids. *J. Pharm. Sci.* 79:992–998 (1990).