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Low Molecular Weight Heparins Cross Rat Gastric Mucosa Mounted in an Ussing Chamber

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Abstract: The aim of this study was to determine if the stomach is a site for low molecular heparin (LMWH) absorption. Gastric mucosa was mounted in a Vertical Diffusion Ussing Chamber and the LMWHs, tinzaparin or reviparin, were added to the mucosal buffer at pH 7.4 or 4.0. Potential difference (PD), resistance and short circuit current (Isc) were measured across the mucosa. Buffers and tissues were analyzed for chemical LMWH and anti-factor Xa activity. The PD became more negative on LMWH addition when the mucosal side was compared to the serosal side. The PD returned to baseline following a lag period, which was greater at pH 4.0 versus 7.4. Changes in resistance were similar to those for PD. Isc increased with time at pH 7.4 but not pH 4.0, which was most dramatic for reviparin. LMWHs were recovered from serosal buffer and tissue and had anti-factor Xa activity at both pH 7.4 and 4.0 although amounts found in serosal buffer and rate of movement were greater at pH 7.4 versus 4.0. In conclusion, changes in PD, LMWH recovery and anti-factor Xa activity in serosal buffer indicate that LMWHs cross rat gastric mucosa. Changes in Isc suggest that active transport may depend on mucosal pH. Furthermore, LMWHs cross gastric mucosa under both neutral and acidic conditions, however transport is faster in a neutral environment suggesting that the stomach, with an acidic environment, may not be the main site for LMWH absorption *in vivo*.

Key words: Low molecular weight heparin, oral, stomach, rats, absorption

INTRODUCTION

Significant patient morbidity and mortality, due to thrombosis, is a major health concern (Hirsh *et al*., 1995). Low molecular weight heparins (LMWHs) given by intermittent subcutaneous injection are safe and effective antithrombotic drugs in both the in-hospital and out-of-hospital setting. Oral administration of LMWHs is not considered effective. This is due to studies showing little evidence of bioavailability of oral LMWH and similar polyanionic compounds measured by anti- factor Xa activity or the activated partial thromboplastin time (APTT) (Iqbal *et al*., 2001; Lorentsen *et al*., 1989; Faaij *et al*., 1999; Salartash *et al*., 2000).

In opposition to these studies, findings from our laboratory and others have clearly demonstrated that low molecular weight polyanions such as dextran sulfate, sulodexide, pentosan polysulfate, sucrose octasulfate and LMWHs are absorbed following oral administration (Jaques *et al*., 1991; Ofosu, 1998; Nickel *et al*., 2000; Hiebert *et al*., 1999, 2000, 2001). Low molecular weight polyanions such as sucrose octasulfate and dextran sulfate were found associated with endothelium following oral administration despite little evidence of their presence in plasma (Jaques *et al*., 1991; Hiebert *et al*., 2002). Moreover, the LMWHs tinzaparin and reviparin and dextran sulfate, when given orally, have antithrombotic activity in a rat jugular vein thrombosis model (Jaques *et al*., 1991; Hiebert *et al*., 2000, 2001).

Although evidence for oral absorption of LMWHs exists in the literature, there are no studies concerning the site of absorption. Tissue distribution studies of tinzaparin, following administration to rats by the oral route, show progression through the gut with peak amounts of the drug recovered from stomach tissue and washes at 6-30 min of administration compared to 15-30, 30 min, 2 and 4 h, for duodenum, jejunum, ileum and colon, respectively (Hiebert *et al*., 2004). The amount of tinzaparin in stomach at 15 min accounted for 46% of the drug administered versus 0.5% in colon at 4 h suggesting the stomach was an important distribution site. These findings agree with investigations of tissue distribution of [^{14}C]-labeled and cold sucrose octasulfate following oral administration in rats (Hiebert *et al*., 2002) where 84% of the dose administered was found in the stomach tissue at 6 min. Thus the stomach may be a site of absorption of LMWHs.

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In our most recent *in vitro* study using a vertical diffusion Ussing chamber, we observed that the stomach could be a site for absorption of unfractionated heparin (UFH) by indicating that bovine UFH moves across rat gastric mucosa from the mucosal side (lumen) to the serosal side (circulation) in both a dose- and pH-dependent manner (Moazed and Hiebert, 2007). UFH consists of polydisperse chains of varying molecular weight from 500 to over 40,000 Daltons (Da) (Linhardt and Gunay, 1999). In contrast, LMWHs consist of only short chains of less than 8000 Da. LMWHs are obtained from UFH by various methods of fractionation or depolymerization and each LMWH is unique in structure (Linhardt and Gunay, 1999; Hirsh *et al*., 1998). While UFH has both anti-factor Xa and IIa anticoagulant activity, LMWHs have primarily anti-factor Xa activity (Johnson *et al*., 1976; Andersson *et al*., 1976). LMWHs also differ from UFH in their half-life, tissue binding, clinically effective doses, side effects observed and choice of effective neutralizing agents (Hirsh *et al*., 1998; Hirsh *et al*., 2001). LHWHs are now widely used as antithrombotic agents. Since LMWHs have both different chemical structure and biological effectiveness than UFH, information on the site of their possible absorption may differ from UFH. The main objective of this study was to investigate if LMWHs cross rat gastric mucosa, mounted in an Ussing chamber and to better understand the site of absorption of LMWHs by manipulating the pH of the mucosal buffer.

MATERIALS AND METHODS

This study was performed in the Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Canada in 2005-2006.

Animals: Twenty-four male Wistar rats weighing between 250 and 300 g were used in the study. Animals were obtained from the Charles River Canada Company, St. Constant, Quebec, Canada and were handled in accordance with the Principles of Animal Care set by the Canadian Federation of Biological Societies. All animal procedures were approved by the Animal Care Committee of the University of Saskatchewan and performed according to the guiding principles of the Canadian Council on Animal Care.

Isolation of gastric mucosa: Animals were anesthetized by injection of Equithesin (chloral hydrate 4.2% w/v, sodium pentobarbital 0.98% w/v, magnesium sulfate 2.12% w/v, ethanol 10% v/v, propylene glycol 40% v/v and sterile water to a volume of 100 mL: 1 mL/250 g rat) through the intraperitoneal route. The abdominal cavity was entered by a medio ventral incision. The stomach was removed, placed in saline and opened from the lesser curvature. It was then washed several times with saline until all contents and blood were removed. The stomach was then stretched, mucosal side up, on the cork bottom of a plastic tray placed on ice, using pins to hold it in place. The mucosa of the glandular portion of the stomach was carefully stripped from submucosa and serosa using a scalpel blade, leaving an intact mucosa for use in the Ussing chamber.

Measurements of electrical parameters across mucosa using an Ussing chamber: An EVC 4000 voltage/current clamp (NaviCyte, Harvard Apparatus, Inc.) was used for transport studies and included: a vertical diffusion Ussing chamber made of two hemi-chambers; electrodes for sensing the voltage and for passing current and instrumentation for measuring voltage and current. Immediately after separation, the mucosa was mounted on the pins of one hemi-chamber and the matching hemi-chamber was attached to seal the diffusion apparatus. Thus two separate compartments, the mucosal compartment on the mucosal side and serosal compartment on the serosal side of the mucosa were created. As a control, an additional portion of the mucosa was frozen for later glycosaminoglycan (GAG) extraction.

The assembled chamber was placed in a block heater connected to a circulating water bath maintained at 37°C. The hemi-chambers were filled with 1.5 mL of warmed (37°C) oxygenated Kreb's Ringer bicarbonate buffer ($MgCl_2.6H_2O$, 1.1 mM; $CaCl_2.2H_2O$, 2.15 mM; NaCl, 113.96 mM; KCl, 5.03 mM; Na_2HPO_4, 1.65 mM; $NaH_2PO_4.H_2O$, 0.30 mM; $NaHCO_3$, 25 mM at pH 7.4 or 4.0). D-glucose (40 mM) was added to the serosal buffer to provide an energy source. Mannitol (40 mM) was added to the mucosal buffer to provide an osmotic load equal to the serosal buffer. Buffers were added to each side of the chamber simultaneously to prevent hydrostatic pressure effects. Buffers in the hemi-chambers were circulated by gas lift (95% O_2/5% CO_2), controlled by valves (Precision Instrument Design, Los Altos, CA). The exposed mucosal surface area was 2.5 cm^2.

Harvard/Navicyte Micro-Reference voltage measuring electrodes (2.5 mm × 5.0 cm) and electrodes for passing current were placed on either side of the mucosa as previously described by Moazed and Hiebert (2007). After eliminating asymmetries in the voltage measuring electrodes, the following electrical properties across the mucosa were measured at specific intervals: potential difference (PD) or voltage difference across the mucosa (ΔV) in mV, resistance (R) in mΩ and short circuit current (Isc) in mA (a measure of the net active ion transport across the mucosa). To measure these properties, a

current of 15 mA was passed across the mucosa using the pulse generator and V was recorded. The R was then calculated using Ohm's law: $R = \Delta V_t I^{-1}$, where ΔV_t is voltage difference across the mucosa and I is the current of 15 mAmps. The transmucosal current was then clamped to zero (i.e., no net transmucosal current flow) and ΔV_t was measured. Since R of the tissue is known, the short circuit current (Isc) can then be calculated: $Isc = \Delta V_t R_t^{-1}$

The tissue was stabilized in buffer for 40 min with electrical measurements taken every five min. The LMWHs were then added to the mucosal buffer by adding 0.1 mL of the LMWH stock solutions to obtain a final concentration of 10 mg mL^{-1}. Electrical measurements were continued every two min for an additional 84 min. Mucosal and serosal buffers as well as mucosal tissues were then collected and frozen at -4°C for later extraction and analysis. Electrical parameters, PD, R and Isc, were normalized to the value taken just prior to LMWH addition at 40 min. Changes in PD, R and Isc were then determined by subtracting all recorded values at all other times during the experiment from the recorded value at 40 min.

Assessment of mucosal injury: To determine any existing minor damage in the isolated gastric mucosa, mucosal tissue was mounted in the Ussing chamber and tinzaparin was added to the mucosal buffer. The experiment was performed for 124 min as described above. Then, 100 μL of trypan blue stock solution (0.3 mg mL^{-1}) was added to the mucosal buffer. After 15 min, mucosal and serosal buffers were collected and absorbance was read at 590 nm. Serosal buffer, without trypan blue addition, was used as a control. The trypan blue concentration in samples was determined by comparing the absorbance to a reference curve prepared with known amounts of the dye.

LMWH extraction from buffers and tissue: Mucosal and serosal buffers were dialyzed in distilled water for 48 h using MWCO 1000 dialysis tubing. The dialyzed buffers were then dried and used to analyze chemical heparin and anti-Xa activity. GAGs were extracted from mucosal tissue by a published method with modifications (Jaques, 1977). Mucosa was minced, defatted with acetone and isopropanol/petroleum ether (1:1) and digested by protease in 0.1M Tris buffer with 0.1M $CaCl_2$ at pH 8.0 at 37°C. Digests were purified by precipitating with 1% NaCl in acetone and methanol. The precipitates were then dried.

Identification and chemical measurement of extracted LMWH: Agarose gel electrophoresis was used to identify and measure LMWH in extracts. Dried powders were dissolved in suitable volumes of water and applied to agarose gel slides, along with the administered LMWH as a reference. Gels were fixed in 0.1% hexadecyltrimethylammonium bromide and air-dried. Slides were stained with 0.04% toluidine blue in 80% acetone and background color was removed using 1% acetic acid. The LMWH was identified by electrophoretic migration as compared to reference material and amounts were determined by densitometry.

Measurement of anti-factor Xa activity in buffers: Mucosal and serosal buffer extracts were desalted using QIAprep® Spin M13 Kit columns. Columns were washed twice with distilled water by centrifuging at 5000 rpm for 5 min. Dried powders from dialyzed buffers were dissolved in suitable volumes of distilled water (10 mL and 100 μL for mucosal and serosal buffer extracts, respectively). Solutions were then added to the washed columns and after 15 min, columns were washed twice with distilled water and centrifuged at 5000 rpm for 5 min. The membrane of the spin column was then washed with 200 μL of distilled water using a pipette and the washes were collected and dried obtaining a desalted buffer extract.

A chromogenic assay was used to identify and measure anti-Xa activity in the desalted buffer extracts. Human antithrombin III (200 μL) was added to a plastic test tube. Next, 25 μL of LMWH standard or buffer extracts was added, mixed and incubated at 37°C for 2 min. Bovine factor Xa (200 μL) was then added with mixing and incubated at 37°C for 1 min. Factor Xa substrate (200 μL) was added to the same tube and incubated for exactly 5 min. Finally, 200 μL acetic acid was added with mixing to terminate the reaction. Absorbance of the solution was read at 405 nm. Concentrations of LMWH in samples were determined by comparing the absorbance to a reference curve prepared with known amounts of LMWH.

Drugs and chemicals: Tinzaparin (tinzaparin sodium, anti-Xa activity 90.7 IU mg^{-1}, peak maximum molecular mass of 5600 daltons) obtained from porcine mucosal heparin, was generously donated by Novo Nordisk, Denmark. Reviparin (reviparin sodium, Batch W 49522, average molecular mass of 4300 daltons, anti-Xa activity 130 IU mg^{-1}; anti-IIa activity 29 IU mg^{-1}) was from Knoll AG, Ludwigshafen, Germany. Both LMWHs were prepared as a stock solution of 150 mg mL^{-1} in Kreb's buffer. Materials for gel electrophoresis; petroleum ether, glacial acetic acid and acetone were obtained from VWR Canlab, Mississauga, ON, Canada; sodium barbital, cetavlon (hexadecyltrimethylammonium bromide), toluidine blue and HCl were from Sigma-Aldrich Canada Ltd, Oakville, ON, Canada and agarose was from Bio-Rad, Mississauga, ON, Canada. Materials for Kreb's buffer,

$MgCl_2.6H_2O$, $CaCl_2.2H_2O$, NaCl, KCl, Na_2HPO_4, $NaH_2PO_4.H_2O$, $NaHCO_3$, mannitol, D-glucose, were from VWR Canlab. Molecular weight cut off (MWCO) 1000 dialysis tubing was purchased from Spectrum Laboratories Inc., RanchoDominguez, CA, USA. Materials for LMWH extraction from mucosal tissues; protease from Streptomyces griseus was from Sigma-Aldrich; Tris, $CaCl_2$, isopropanol and methanol were from VWR Canlab. The chromogenic assay kit (Accucolor™ Heparin®) for measurement of anti-Xa activity was obtained from Sigma-Aldrich. QIAprep® Spin M13 Kit columns for desalting the buffers were from Qiagen, Mississauga, ON, Canada. Chemicals for anesthesia, chloral hydrate, sodium pentobarbital, magnesium sulfate, ethanol and propylene glycol were obtained from Sigma-Aldrich.

Statistics: All data are expressed as Mean±standard error of the mean (SEM). A one-tailed unpaired t-test was used to determine significant differences in the lag period, to compare differences in the time taken for PD to reach the resting level after the lag period in different environments and to measure differences in PD increase upon heparin addition to the mucosal side of the Ussing chamber. A one-tailed t-test was also used to measure differences in LMWH concentrations in serosal buffers and experimental mucosa and to compare rates of movement of each LMWH across the mucosa under different mucosal buffer pH conditions.

A two-tailed t-test was used to compare differences in LMWH concentrations in serosal and mucosal buffer extracts based on anti-Xa activity and rates of movement of different LMWHs across the mucosa under the same mucosal buffer pH conditions. A paired two-tailed t-test was used to measure differences in trypan blue concentrations between mucosal, experimental and control serosal buffers. A two-tailed t-test, non-parametric Mann-Whitney test, was used to compare differences in Isc during the experimental period between groups. Differences in Isc were calculated by subtracting the first 5 values after LMWHs addition, obtained at 40-48 min, from the last 5 values recorded, obtained at 116-124 min, during the experimental protocol. Values were considered significant at $p<0.05$.

RESULTS

Addition of tinzaparin to mucosal buffer at pH 7.4: The gastric mucosa was placed in the Ussing chamber with buffers at pH 7.4 and electrical parameters were recorded every 5 min. Following tissue stabilization for 40 min, tinzaparin (10 mg mL^{-1}) was added to the mucosal buffer and electrical parameters were measured every 2 min for an additional 84 min. When tinzaparin was introduced into the mucosal buffer, the charge difference between the mucosal and serosal side of the membrane increased as the PD became negative compared to that prior to addition (Fig. 1a). After a lag period of 7.0±1.2 min, the PD began to decrease. The PD achieved the previous resting level 35.0±9.5 min later. Changes in R followed a pattern similar to that seen for PD (Fig. 1b). The R decreased upon addition of tinzaparin and following a lag period of 8.0±1.4 min returned to the resting level 32.0±2.5 min later. The Isc showed a slight increase of 2.7±0.5 mA throughout the experimental period (Fig. 1c). Placing buffer instead of tinzaparin into the mucosal side of the Ussing chamber caused no change in PD, R or Isc across the membrane (Fig 1a- c).

At the completion of the experiment, tinzaparin was found in the serosal buffer (176.8±75.7 μg mL^{-1}) and the experimental mucosal tissue (25.0±1.5 μg g^{-1}) as well as in mucosal buffer (Table 1, Fig. 2). Tinzaparin was not found in control mucosal tissue. Based on the recovery from the serosal buffer, the calculated rate of movement of tinzaparin across the mucosal tissue was 1.26±0.54 μg/cm^2/min.

Addition of tinzaparin to mucosal buffer at pH 4.0: To mimic the *in vivo* pH of the stomach, rat gastric mucosa was placed in the Ussing chamber with mucosal buffer at pH 4.0, the average pH in rat stomach. When tinzaparin was added to the mucosal buffer, PD increased and became more negative as in the neutral condition. After a lag period, the PD began to decrease to the previous resting level (Fig. 3a, Table 3). There was a doubling of the lag period in the acidic compared to the neutral environment, 16.4±0.5 min compared to 7.0±1.2 min, respectively although the difference did not reach statistical significance ($p = 0.2$, one-tailed t-test). PD reached the previous resting level 47.6±11.4 min after the lag period in the acidic environment compared to 35.0±9.5 min at pH 7.4 ($p = 0.2$, one-tailed t-test). The Isc increased throughout the experimental period (40-124 min) by 0.9±0.5 mA at pH 4.0 compared to 2.7±0.5 mA at pH 7.4 ($p = 0.06$, two-tailed t-test, Mann-Whitney test) (Fig. 3b, Table 3).

Tinzaparin recovery from the serosal buffer was 51.5±2.5 μg mL^{-1} when added to the mucosal buffer at pH 4.0, compared to 176.8±75.7 μg mL^{-1} when added at pH 7.4 ($p = 0.06$, one-tailed t-test). Furthermore, 34.2±5.2 μg g^{-1} tinzaparin was recovered from the mucosal tissue following addition to the mucosal buffer at pH 4.0 compared to 25.0±1.5 μg g^{-1} when added at pH 7.4 ($p = 0.04$, one-tailed t-test) (Table 1). The calculated rate of movement of tinzaparin across the mucosal tissue at pH 4.0 was 0.40±0.02 μg/cm^2/min compared to 1.26±0.54 μg/cm^2/min at neutral pH ($p = 0.06$, one-tailed t-test).

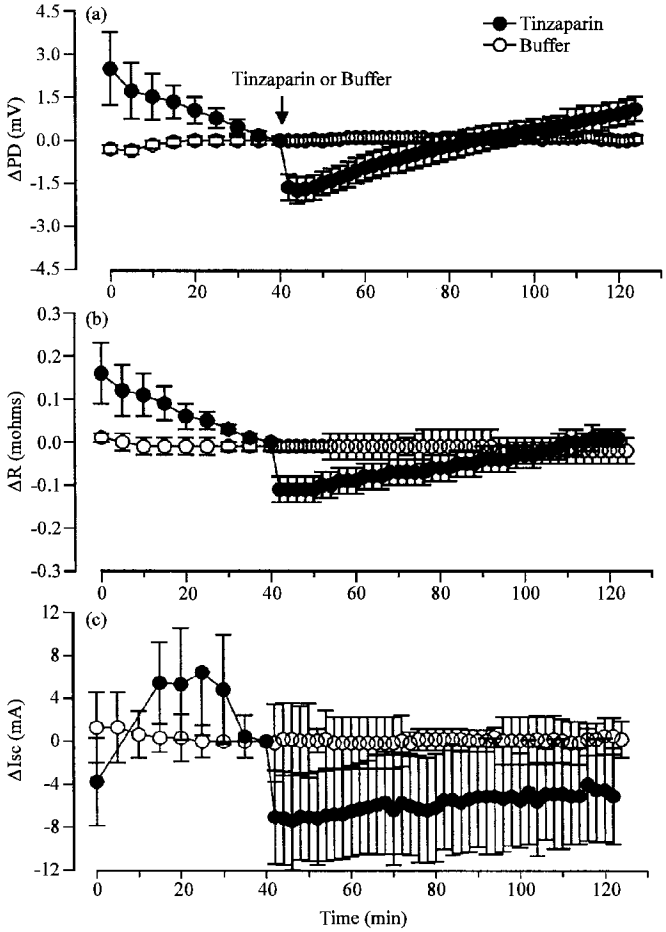

Fig. 1: Changes in electrical parameters across rat gastric mucosa on tinzaparin (10 mg mL^{-1}) or Kreb's buffer addition to the mucosal buffer at neutral pH. The potential difference (PD) became more negative when the mucosal side was compared to the serosal side immediately after addition of tinzaparin to the mucosal buffer. PD returned to the resting level with time (a). Changes in resistance (R) are similar to the pattern of changes in PD (b). Short Circuit Current (Isc) showed a slight but non significant increase during 84 min of the experiment (c). No change was observed in PD (a), R (b), or Isc (c) if buffer alone was added to the mucosal side. Results are shown as Mean±SEM of 7 experiments for tinzaparin and 3 experiments for buffer

Table 1: Tinzaparin and Reviparin recovered from buffers and tissues 84 minutes after addition to the mucosal buffer when rat gastric mucosa was placed in an Ussing chamber

Treatments	Mucosal buffer (μg mL^{-1})	Serosal buffer (μg mL^{-1})	Control mucosa (μg g^{-1})	Experimental mucosa (μg g^{-1})
Tinzaparin (pH 7.4) 10 mg mL^{-1}, (n = 7)	2266.7±584.2	176.8±75.7	0.0±0.0	25.0±1.5
Tinzaparin (pH 4.0) 10 mg mL^{-1}, (n = 6)	3250.0±845.6	51.5±2.5	0.0±0.0	34.2±5.2[a]
Reviparin (pH 7.4) 10 mg mL^{-1}, (n = 4)	2875.0±953.8	214.1±70.6[b]	0.0±0.0	24.4±1.8
Reviparin (pH 4.0) 10 mg mL^{-1}, (n = 4)	3500.0±333.3	47.5±5.0	0.0±0.0	35.0±8.5

Results are shown as Mean±SEM. [a]Significantly greater than tinzaparin at pH 7.4, one-tailed t-test. [b]Significantly greater than reviparin at pH 4.0, one-tailed t-test

Anticoagulant activity of tinzaparin in buffers: To determine if tinzaparin recovered in the serosal buffer had anticoagulant activity, anti-Xa activity was measured in the desalted buffer extracts when added to mucosal buffer at pH 4.0. Anti-Xa activity was found both in the mucosal and serosal buffer extracts. Tinzaparin concentration

Table 2: Trypan blue recovered from buffers 15 min after addition to the mucosal buffer when rat stomach mucosa was placed in an Ussing chamber for 124 min

Buffers	Trypan blue (mg mL^{-1})
Mucosal buffer	53.3±5.7
Experimental serosal buffer*	1.5±0.1
Control serosal buffer**	1.6±0.3

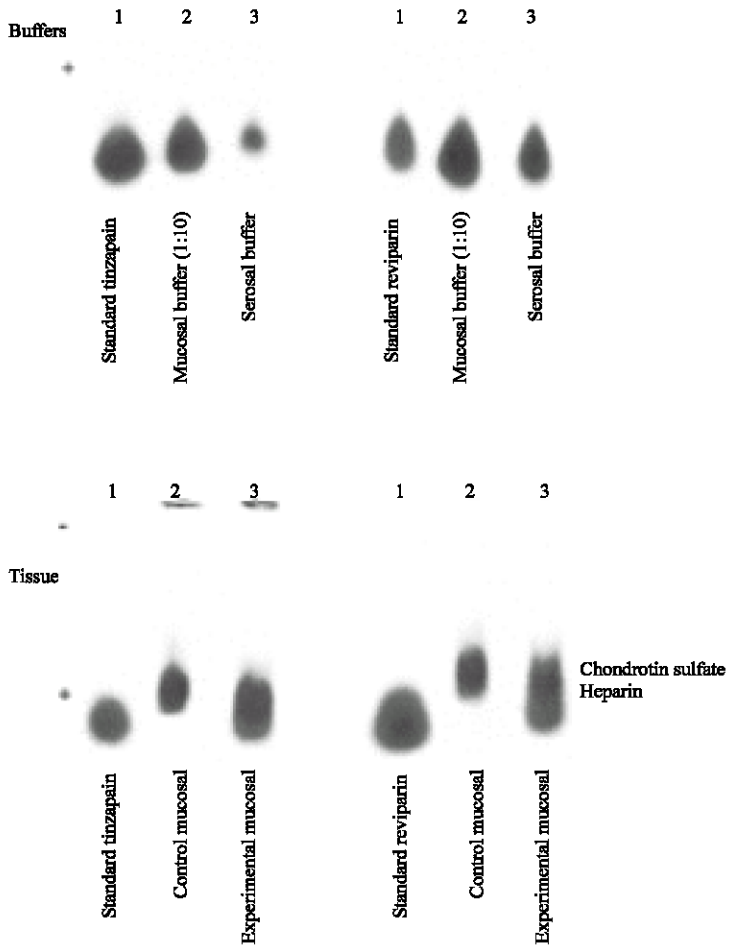

Fig. 2: An electrophoretic gel showing low molecular weight heparin recovery from buffers and tissues following addition to the mucosal buffer. Using agarose gel electrophoresis, low molecular weight heparins (LMWHs) were found in both the mucosal and serosal buffer as well as in experimental tissue 84 min after their addition to the mucosal buffer. LMWH standard and buffer and tissue extracts were dissolved in measured amounts of distilled water and 2 μL was applied to each lane (level with the negative sign). The upper gels show LMWHs (tinzaparin or reviparin) recovered from serosal buffer extracts: Lane 1, standard tinzaparin or reviparin 2 mg mL^{-1}. Lane 2, mucosal buffer extract dissolved in 10000 μL of water. Lane 3, serosal buffer extract dissolved in 50 μL of water. The lower gels show LMWHs (tinzaparin or reviparin) recovered from the mucosal tissue: Lane 1, Standard tinzaparin or reviparin 2 mg mL^{-1}. Lane 2, control tissue extract where buffer alone was added to the mucosal side, dissolved in 50 μL of water. Lane 3, experimental mucosal extract dissolved in 50 μL of water. All material extracted from buffers is similar in migration and color to the original standard LMWH. A blue band, moving slower than standard LMWH and likely chondroitin sulfate, is seen in the control mucosa (Lane 2). This band is seen in the experimental mucosa along with LMWH (Lane 3)

based on anti-Xa activity was 13.6±1.2 μg mL^{-1} in serosal compared to 133.2±2.1 μg mL^{-1} in the mucosal buffer extracts.

Assessment of mucosal damage using trypan blue: Trypan blue was used to detect any minor damage to the mucosal tissue that may occur during the process of isolation. Mucosal and serosal buffers were collected 15 min after addition of 100 μL of trypan blue stock solution (0.3 mg mL^{-1}) to the mucosal buffer following experiments lasting 124 min. The concentration of trypan blue in the mucosal buffer was significantly different than that of the serosal buffer, $p<0.0001$, two-tailed t-test. There was no difference in the concentration of trypan blue in the serosal buffer compared to the control buffer ($p = 0.20$, two-tailed t-test) (Table 2).

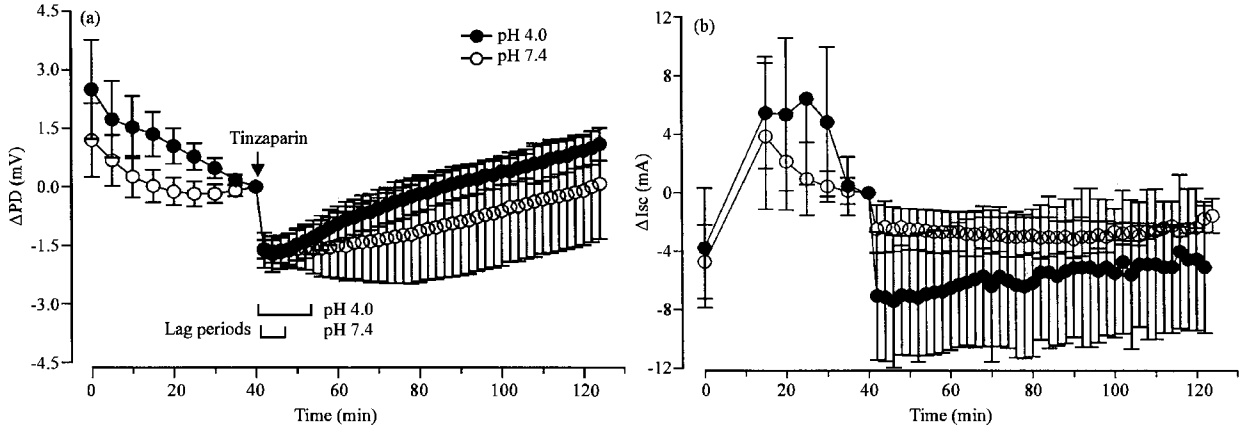

Fig. 3: Changes in electrical parameters across rat gastric mucosa on addition of tinzaparin (10 mg mL^{-1}) to the mucosal buffer at neutral or acidic pH. Tinzaparin was added to the mucosal buffer at pH 4.0 or pH 7.4 and changes in potential difference (PD) and short circuit current (Isc) were compared. The lag period was longer at acidic versus neutral pH (a). Change in Isc was more obvious (124-40 min) after tinzaparin addition at acidic versus neutral pH (b). Results are shown as Mean±SEM of 6 experiments

Table 3: Comparison of changes in electrical parameters (PD, R and Isc) across rat gastric mucosa following addition of tinzaparin or reviparin to the mucosal buffer when mucosa is mounted in an Ussing chamber for 124 min

Electrical parameters		Tinzaparin	Reviparin
pH 7.4	Negativity (mV)	-1.8±0.4	-1.6±0.5
PD	Lag Period (min)	7.0±1.2[a]	4.0±0.0
	Time to reach resting level after lag period (min)	35.0±9.5	36.7±4.3[b]
R	Negativity (mV)	-0.1±0.0	-0.1±0.0
	Lag Period (min)	8.0±1.4	7.3±2.9
	Time to reach resting level after lag period (min)	32.0±2.5	32.7±3.6
Isc	Change in baseline (mA) (124 min-40 min)	2.7±0.5	23.0±12.4[b]
pH 4.0			
	Negativity (mV)	-2.3±0.8	-2.0±0.2
PD	Lag Period (min)	16.4±0.5	15.0±2.8
	Time to reach resting level after lag period (min)	47.6±1.4	46.0±3.7
R	Negativity (mV)	-0.2±0.0	-0.1±0.0
	Lag Period (min)	20.0±9.3	20.0±4.8
	Time to reach resting level after lag period (min)	51.6±12.3	44.5±3.9
Isc	Change in baseline (mA) (124 min-40 min)	0.9±0.5	0.5±2.3

Results are shown as Mean±SEM. [a]Significantly greater than reviparin at pH 7.4. [b]Significantly different than reviparin at pH 4.0

Addition of reviparin to mucosal buffer at pH 7.4 or pH 4.0: To observe if another LMWH caused changes in PD, R, or Isc across rat gastric mucosa, the mucosa was mounted in the Ussing chamber and reviparin was added to the mucosal buffer at pH 7.4 or 4.0. When reviparin was added, the PD became more negative and the charge difference between the mucosal and serosal side of the membrane increased compared to that prior to reviparin addition (Fig. 4a). After a lag period, the PD began to decrease and achieved the previous resting level. There was an increase in the lag period when reviparin was added to the mucosal buffer in the acidic environment versus the neutral environment where lag periods were 15.0±2.8 versus 4.0±0.0 min, respectively ($p = 0.06$, one-tailed t-test, Table 3). The PD reached its previous resting level at 36.7±4.3 min when reviparin was added to the neutral mucosal buffer versus 46.0±3.7 min when added to the acidic mucosal buffer ($p = 0.05$, one-tailed t-test).

Changes in R of the mucosa followed a pattern similar to that seen for PD. The R decreased upon addition of reviparin and then began to increase reaching the resting level 32.7±3.6 min later at pH 7.4 compared to 44.5±3.9 min later at pH 4.0 ($p = 0.03$, one-tailed t-test), following a lag period of 7.3±2.9 min at pH 7.4 compared to 15.0±2.8 min at pH 4.0 ($p = 0.04$, one-tailed t-test) (Results not shown). The Isc increased during the experimental period after reviparin addition with a much more dramatic increase at pH 7.4 (23.0±12.4 mA) than pH 4.0 (0.5±2.3 mA) ($p = 0.05$, two-tailed t-test, Mann-Whitney test) (Fig. 4b, Table 3).

Reviparin was recovered from both serosal buffer and the mucosal tissue. Significantly more reviparin was recovered from serosal buffer when added to the mucosal buffer at pH 7.4 (214.1±70.6 μg mL^{-1}) than at pH 4.0 (47.5±5.0 μg mL^{-1}, $p = 0.02$, one-tailed t-test) (Table 1). Furthermore, 24.4±1.8 μg g^{-1} reviparin was recovered from the mucosal tissue at pH 7.4 compared to

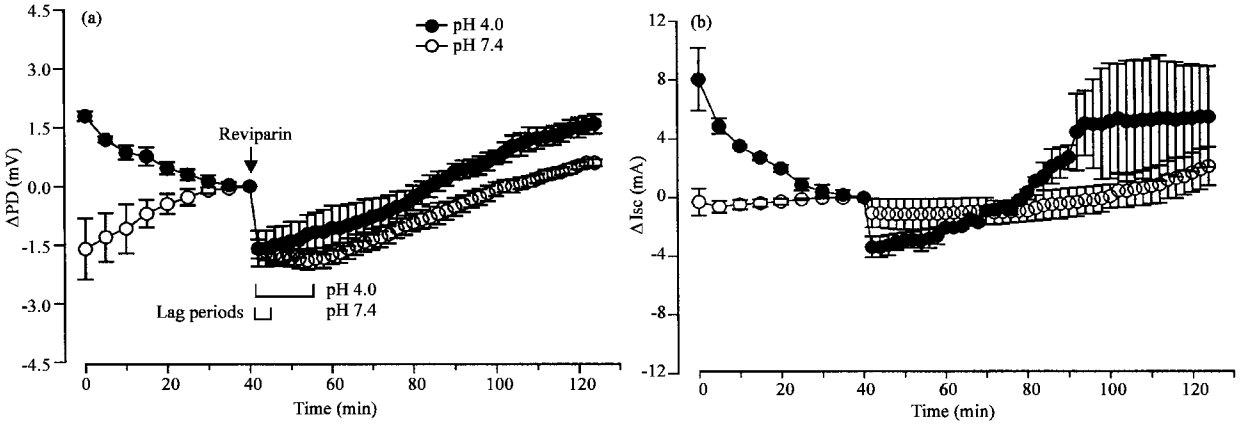

Fig. 4: Changes in electrical parameters across rat gastric mucosa on addition of reviparin (10 mg mL^{-1}) to the mucosal buffer at neutral or acidic pH. Reviparin was added to the mucosal buffer at pH 4.0 or pH 7.4 and changes in potential difference (PD) and short circuit current (Isc) were compared. The lag period was longer at pH 4.0 versus pH 7.4 (a). Short Circuit Current (Isc) showed a dramatic increase at pH 7.4 (124-40 min) but only a slight increase at pH 4.0 (b). Results are shown as Mean±SEM of 4 experiments

35.0±8.5 μg g^{-1} in the acidic environment (p = 0.1, one-tailed t-test) (Table 1). The calculated rate of movement of reviparin across the mucosal tissue was significantly greater at pH 7.4 (1.53±0.51 μg/cm^2/min) compared to pH 4.0 (0.30±0.06 μg/cm^2/min, p = 0.02, one-tailed t-test).

Anticoagulant activity of reviparin in serosal buffers: To determine if reviparin recovered in the serosal buffer had anticoagulant activity, anti-Xa activity was measured in the desalted buffer extracts when reviparin was added to mucosal buffer at pH 4.0. Anti-Xa activity was found both in the mucosal and serosal buffer extracts. Reviparin concentration found in the serosal buffer extracts based on anti-Xa activity was 14.1±1.3 μg mL^{-1} compared to 132.0±5.4 μg mL^{-1} in the mucosal buffer extracts.

Comparison of electrical parameters and recovery following addition of different LMWHs to the mucosal buffer at different pH-pH 7.4: Changes in PD across rat gastric mucosa following addition of reviparin to the mucosal buffer at pH 7.4 were compared to those following addition of tinzaparin. Lag periods for PD were significantly less at 4.0±0.0 min for reviparin compared to 7.0±1.2 min for tinzaparin (p = 0.03, one-tailed t-test, Table 3). Time to return to the previous baseline was 36.7±4.3 min and 35.0±9.5 min for reviparin and tinzaparin, respectively (p = 0.4, one-tailed t-test) (Fig. 5a, Table 3). There was a dramatic difference in changes in Isc following addition of reviparin (23.0±12.4 mA) compared to tinzaparin (2.7±0.5 mA) (p = 0.06, two-tailed t-test, Mann-Whitney test, Fig. 5c and Table 3) where the Isc increased dramatically after addition of reviparin to the mucosal buffer, but showed only a small increase toward the end of the experiment after addition of tinzaparin. Recovery from the serosal buffer and the mucosal tissue was not different after addition of reviparin or tinzaparin. Reviparin recovery was 214.1±70.6 μg mL^{-1} from the serosal buffer compared to tinzaparin recovery of 176.8±75.7 μg mL^{-1} (p = 0.4, one-tailed t-test). Furthermore, 24.4±1.8 μg g^{-1} reviparin was recovered from the mucosal tissue compared to 25.0±1.5 μg g^{-1} tinzaparin (p = 0.4, one-tailed t-test) (Table 1). The calculated rate of movement of reviparin and tinzaparin across the mucosal tissue at pH 7.4 was 1.5±0.5 μg/cm^2/min and 1.3±0.5 μg/cm^2/min, respectively (p = 0.4, two-tailed t-test).

pH 4.0: Changes in the electrical parameters across rat gastric mucosa following addition of reviparin to the mucosal buffer at pH 4.0 were very similar to those following tinzaparin addition. Lag periods for PD were 15.0±2.8 min versus 16.4±0.5 min (p = 0.5, one-tailed t-test) and time to reach the previous resting level was 46.0±3.7 min versus 47.6±1.4 min for reviparin and tinzaparin, respectively (p = 0.5, one-tailed t-test) (Fig 5b, Table 3). The Isc showed a small increase for both reviparin (0.5±2.3 mA) and tinzaparin (0.9±0.5 mA) (p = 1.0, two-tailed t-test, Mann-Whitney test) (Fig. 5d, Table 3). Recovery from the serosal buffers and the mucosal tissues were also very similar with 47.5±5.0 μg mL^{-1} reviparin and 51.5±3.2 μg mL^{-1} tinzaparin recovered from the serosal buffers (p = 0.2, one-tailed t-test) and 35.0±8.5 μg g^{-1} reviparin and 34.2±5.2 μg g^{-1} tinzaparin recovered from the experimental mucosal tissues (p = 0.5, one-tailed t-test, Table 1). Moreover, concentrations of

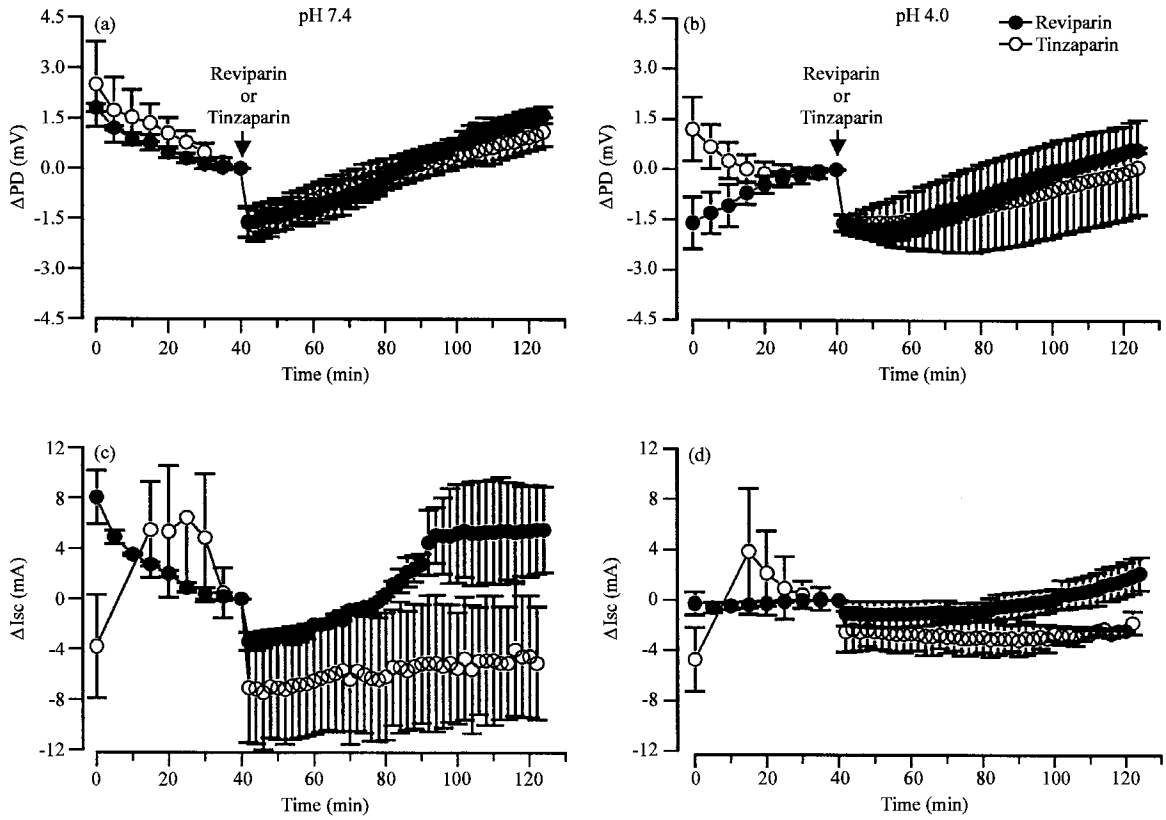

Fig. 5: Comparison of changes in electrical parameters across rat gastric mucosa on addition of tinzaparin or reviparin (10 mg mL^{-1}) to the mucosal buffer at neutral or acidic pH. Rat gastric mucosa was mounted in the Ussing chamber with the mucosal buffer at pH 7.4 or 4.0 and changes in potential difference (PD) and short circuit current (Isc) after addition of tinzaparin were compared to those after reviparin addition. Changes in PD after addition of tinzaparin to the mucosal buffer at pH 7.4 (5a) or pH 4.0 (5b) were superimposed on those following reviparin addition. The Isc showed a dramatic increase at pH 7.4 after reviparin addition to the mucosal buffer versus a small increase for tinzaparin (5c). Unlike tinzaparin, a slight increase in Isc was observed at pH 4.0 for reviparin (5d). Results are shown as Mean±SEM of 4 experiments

reviparin and tinzaparin in the mucosal and serosal buffer extracts based on anti-Xa activity were similar. Reviparin concentration found in the serosal buffer extracts based on anti-Xa activity was 14.1±1.3 μg mL^{-1} compared to 13.6±1.2 μg mL^{-1} for tinzaparin ($p = 0.7$, two-tailed t-test). Reviparin concentration found in the mucosal buffer extracts based on anti-Xa activity was 132.0±5.4 μg mL^{-1} compared to 133.2±2.1 μg mL^{-1} for tinzaparin ($p = 0.8$, two-tailed t-test). The calculated rate of movement of reviparin (0.3±0.1 μg/cm^2/min) and tinzaparin (0.4±0.0 μg/cm^2/min) across the mucosal tissue at pH 4.0 did not differ ($p = 0.14$, two-tailed t-test).

DISCUSSION

Although antithrombotic activity of orally administered LMWHs in rat models has been thoroughly studied, the site of LMWH absorption is unknown. Since LMWHs are similar to endogenous compounds and are difficult to label (Hiebert *et al*., 2000), we have chosen to use the vertical diffusion Ussing chamber in our present study. Since gastrointestinal epithelia are polar in nature, our present study requires the separation of the apical and basolateral side of the gastric mucosa, which is possible when using the Ussing chamber. The Ussing chamber has previously been found useful for transport and drug absorption studies using excised tissue and monolayers of cells grown on filters (Ayalon *et al*., 1982; Yamashita *et al*., 1997; Wallon *et al*., 2005). Furthermore, it has proved to be effective in screening and predicting drug absorption in humans (Yamashita *et al*., 1997; Watanabe *et al*., 2004).

Our current results indicate that the stomach could be a possible absorption site for LMWHs. Both LMWHs,

tinzaparin and reviparin, were recovered from serosal buffer and mucosal tissue after addition to the mucosal side of gastric mucosa in the Ussing chamber. Additionally, anticoagulant (anti-factor Xa) activity of LMWHs was maintained after crossing the mucosa. Changes in electrical parameters also support this observation. The increase in PD negativity on addition of LMWHs to the mucosal buffer is likely due to the high negative charge of LMWHs which make the mucosal side more negative than the serosal. The lag period, when PD does not change, can be explained as the time required for LMWHs to cross the mucosa. The PD then decreases in negativity as the serosal buffer contains more LMWHs. These changes were not artifacts of mucosal damage since trypan blue was not recovered from serosal buffer after its addition to mucosal buffer (Table 2).

Absorption of LMWHs is supported by earlier studies. Intragastric administration of tinzaparin or reviparin significantly reduced thrombotic incidence in a rat jugular vein thrombosis model, in a dose-dependent manner, 4 h after administration (Hiebert *et al*., 2000, 2001). Tissue distribution studies showed that oral tinzaparin and [^{14}C]-labeled and cold sodium sucrose octasulfate, were found in both gut and non-gut tissues. More drug was recovered from stomach tissue compared to lower gut levels and drug was found in plasma at 6 min suggesting absorption in the stomach (Hiebert *et al*., 2002, 2004). Blood clotting time was also prolonged after placing LMWH in the buccal cavity of mice (Lasker, 1975). Similarly, increased blood anticoagulant effect was observed with lower molecular weight fractions, when heparin was separated with ethanol into five molecular weight fractions and introduced into the duodenum of mice with citric acid (Sue *et al*., 1976). As well, LMWHs given in drinking water returned systolic blood pressure of spontaneously hypertensive rats to normal (Vasdev *et al*., 1994).

Differences were seen between the LMWHs. A significantly shorter lag period for PD and a faster rate of movement across the mucosa, based on recovery from serosal buffer (Table 1), was observed for reviparin compared to tinzaparin suggesting a faster rate of absorption for reviparin. In support, the same significant decrease in thrombotic incidence was achieved at a lower oral dose of reviparin (0.025 mg kg^{-1}) compared to tinzaparin (0.1 mg kg^{-1}) in a rat jugular vein thrombosis model (Hiebert *et al*., 2000, 2001). These differences are not surprising since each LMWH is chemically unique depending on the manufacturing process used. LMWHs have specific oligosaccharide composition and microstructural differences (Fareed *et al*., 2004a, b) that may influence drug interaction with gastric mucosa. Tinzaparin is of higher molecular weight (MW) than reviparin and has a 4,5 unsaturated uronic acid at its non-reducing terminus while reviparin has a 2,5 anhydro-D-mannose at the reducing terminus (Fareed *et al*., 2003). The decreased MW of reviparin versus tinzaparin may be a factor contributing to its faster movement through the mucosa. Earlier studies indicate that a decrease in MW facilitates heparin movement through the intestinal membrane (Sue *et al*., 1976).

Changes in electrical parameters indicate that both the lag period and the time required to reach baseline for PD and R, are greater at pH 4.0 compared to pH 7.4 (Table 3). This suggests a slower movement of LMWHs across the gastric mucosa at lower pH, supported by decreased recovery of LMWHs in serosal buffer and a slower calculated rate of movement across the mucosa at pH 4.0 than 7.4. Therefore more LMWHs may be absorbed in the small intestine where pH is higher compared to the stomach. Since LMWHs have little capacity for protein binding, they may pass easily through the stomach lumen, entering the more basic duodenum.

Although this study was not designed to investigate the mechanism of absorption, our results suggest the involvement of both active transport and passive diffusion. The Isc increased over time at pH 7.4 but not pH 4.0. Since change in Isc is an indicator of active transport (Cooke and Dawson, 1978; Chao *et al*., 1990; Hurst *et al*., 1995), active transport and passive diffusion may be responsible for the movement of LMWHs across the mucosa at pH 7.4 and 4.0, respectively. Transporters for anionic compounds may become activated under neutral or more basic conditions. Changes in Isc are more dramatic for reviparin than tinzaparin at pH 7.4. This may be explained by separate transporters of varying numbers for anionic compounds of different MWs. Reviparin may have a greater affinity for a transporter than tinzaparin, or reviparin once bound to the receptor may interact with it in a more rapid reversible manner. Changes in R also indicate involvement of passive transport. When the mucosal buffer LMWH concentrations are high, R is decreased and the concentration gradient across the mucosa helps LMWH cross the membrane. As LMWH concentrations decrease, the concentration gradient across the mucosa decreases and R increases.

CONCLUSION

These results support earlier studies that LMWH is absorbed following oral administration. Results of this study suggest that LMWHs cross the stomach tissue, however movement is faster at neutral pH than under the

natural acidic conditions found in the stomach. Although LMWH may be absorbed in the stomach, the intestine at neutral pH may be the preferred site of absorption. Further studies are required to investigate the mechanism of LMWHs movement across the gastrointestinal mucosa and to evaluate the role of passive and active transport in the process.

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