Heparin Absorption Across the Intestine: Effects of Sodium *N*-[8-(2-Hydroxybenzoyl)Amino]Caprylate in Rat *In Situ* Intestinal Instillations and in Caco-2 Monolayers

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Purpose. The effects of sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC) on heparin intestinal absorption were studied using rat *in situ* ileal and colonic instillations and Caco-2 monolayers.

Methods. The flux of heparin was tested in the following groups: i) heparin alone, ii) heparin in the presence of SNAC, iii) heparin in the presence of propylene glycol (PG), and iv) heparin in the presence of SNAC and PG. Heparin absorption was measured by the APTT assay in the *in situ* models and by the anti-Factor Xa assay in Caco-2. SNAC and [^{3}H]-SNAC fluxes were assessed by HPLC and by scintillation counting respectively.

Results. In the rat ileal and colonic *in situ* instillations SNAC (17–35mg) promoted heparin absorption in the presence and absence of PG without damaging the tissue. PG alone did not alter heparin absorption *in situ*, but it amplified the effect of SNAC. In Caco-2, enhanced heparin fluxes were variable in the presence of non-cytotoxic concentrations of SNAC (<10mg/ml) and these effects could not be discriminated from those of PG. Papp values for SNAC alone were 2.2×10^{-5} cm/s and 2.0×10^{-5} cm/s in the mucosal-to-serosal and serosal-to-mucosal directions respectively, suggesting a substantial passive transcellular flux. Transport of SNAC was significantly reduced in the presence of heparin and/or PG, perhaps indicating physical association between the agents.

Conclusions. SNAC augmented heparin absorption alone and in combination with PG in the rat *in situ* models without causing toxicity. Caco-2 had limitations for testing increased heparin absorption due to cytotoxic effects of high concentrations of SNAC and PG. However, SNAC itself was well absorbed across Caco-2 and its mechanism of permeation was determined.

KEY WORDS: heparin absorption; amido acid; rat intestinal instillation; Caco-2.

INTRODUCTION

Intravenous and subcutaneous administration of unfractionated heparin (molecular weight, 12–20kD) is indicated as a post-operative treatment for patients at risk from deep vein thrombosis and pulmonary embolism (1,2). Patients are usually switched from intravenous or subcutaneous heparin to oral warfarin upon hospital discharge. Heparin causes a rapid onset of anticoagulant activity and has a short half life in plasma (3). In contrast warfarin has a slow onset, is predominantly protein bound, is subject to drug-drug interactions and requires careful monitoring (4). Although both agents are effective anti-coagulants, heparin is pharmacologically superior to warfarin (reviewed in 3).

An oral heparin formulation is the preferred warfarin replacement option for out-patient therapy because monitoring can be carried out by the simple and rapid activated partial thromboplastin time (APTT) assay. To date, oral administration of the glycosaminoglycan has not been possible because heparin tends to desulphate and to undergo glycoside metabolism under the acidic conditions of the stomach (5). Also, heparin absorption across the epithelium of the gastrointestinal tract is inherently very poor, similar to that of peptides of similar size (reviewed in 6). A possible oral formulation of heparin might therefore include protection against stomach acid and the enzyme activity of the upper gastrointestinal tract. Assuming that this is feasible, we have focused on the subsequent step, namely to increase heparin flux across the small intestine and colon by co-administering an absorption promoter.

Sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC) was selected as a candidate to increase the intestinal absorption of heparin. Previously, *N*-acylated amino acid derivatives were shown to facilitate cromolyn permeation across rat intestinal mucosae *in vitro* and after oral gavage (7). Benzoylated and phenylsulphonated amino acids also showed potential for the oral delivery of calcitonin and α-interferon (8) and for rhGH (9) in rats and primates. Several derivatized non-α-amino acids from this class have also been shown recently to enhance absorption of heparin after oral administration to rats and primates (10). From this class of compounds SNAC has emerged as a lead candidate for development as an oral heparin delivery agent. With a molecular weight of 303 for the sodium salt, it has an apparent log octanol/water partition coefficient of 0.7 and an apparent pKa of 5.2 (11). Its drug delivery characteristics are based on proteinoid technology for drug delivery (12,13). Unlike the previous generations of this class of drug delivery agent, SNAC can facilitate drug absorption in solution at neutral pH without the requirement for microsphere formation. The precise mechanism by which by these delivery agents effect drug permeation is the subject of continuing studies (10) and is also addressed here.

Heparin absorption in the presence of SNAC was studied using rat *in situ* ileal and colonic instillations and Caco-2 monolayers. Fluxes were also tested with propylene glycol (PG), a molecule which has potential as a formulation excipient. The extent and mechanism of absorption of SNAC and [^{3}H]-SNAC across Caco-2 were determined and a preliminary assessment of SNAC metabolism during intestinal transport was made. The results showed that SNAC significantly elevated heparin bioavailability in the rat *in situ* models without damaging the tissue, whereas the results in Caco-2 were inconclusive due to the cytotoxic effects of high concentrations of SNAC.

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ABBREVIATIONS: Papp (apparent permeability coefficient); APTT (activated partial thromboplastin time); PG (propylene glycol); SNAC (sodium *N*-[8-(2-hydroxybenzoyl) amino]caprylate); TER (transepithelial resistance); DMEM (Dulbecco's Modified Eagles Medium); H & E (haematoxylin and eosin); EM (electron microscopy); and rhGH (recombinant human growth hormone).

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MATERIALS AND METHODS

Materials

USP sodium heparin for Caco-2 fluxes was obtained from Sigma (UK). The sodium salt of SNAC was manufactured by Emisphere Technologies Inc. (US). [3H]-SNAC was made by New England Nuclear (US). The anti-Factor Xa kit (Coatest®) was obtained from Chromogenix AB (Sweden). Caco-2 (passages 18–35) were obtained from ATCC (US). Snapwell Transwell® were obtained from Costar (Netherlands). All tissue culture media were from Gibco (UK). The EndOhm® voltammeter was from W.P.I. (UK). The orbital shaker was an IKA® Schuttler model MTS-2 from Janke-Kunkel (Germany). The diffusion chambers, electrodes, MacLab data acquisition system were from AD Instruments (UK). The BBL Fibrometer was obtained from VWR Scientific, New Jersey (US) and the APTT reagents were purchased from Sigma, Missouri (US). A Biotrack 512 Coagulation Automated Monitor was obtained from Boehringer (Germany). All other chemicals were reagent grade.

In Situ Fluxes Across Rat Ileum and Rat Colon

In situ rat ileal closed loop instillation studies were modifed from the method of Doluisio *et al.* (14). Male or female rats (weight range 230g–330g) were anaesthetised with i.m. ketamine hydrochloride (80 mg/kg)/acepromazine (ACP, 3 mg/kg). A mid-line incision was made and the distal end of the ileum located. 7–9 cm of tissue was isolated and the distal end ligated, taking care to avoid damage to surrounding blood vessels. The proximal end was then ligated following administration of 1 ml/kg of test solution. The loop was sprayed with isotonic saline to provide moisture and then replaced in the body cavity carefully avoiding distension. The incision was closed with standard surgical clips. In separate experiments examining intracolonic dosing of anaesthetised rats, a 7.5 cm 8 fr. Rusch catheter connected to a 1 ml syringe was inserted into the colon through the anus until the tube was no longer visible. The test solution (1 ml/kg) was expressed slowly into the colon via the catheter. Citrated blood samples from all animals were collected from the tail artery at 20 minute intervals for 120 minutes and APTT was measured in the harvested plasma. Some animals were administered 250 I.U. heparin in 0.2 mls by subcutaneous injection as a reference control.

Selected ileal tissues were fixed in 10% neutral formalin, embedded in paraffin wax, microtomed to 5μm sections and conventionally stained with H & E. Independent histological assessment of the tissues was performed by a qualified pathologist at Covance Ltd (UK).

In Vitro Fluxes of Heparin and SNAC Across Caco-2 Monolayers

Caco-2 monolayers were sub-cultured at confluence from 25 cm^2 tissue flasks onto 12mm Snapwell filters (pore size 0.4 μm) at a density of 0.5×10^6 cells/filter according to previous descriptions (15). They were cultured at 37°C with 5% CO_2 in air in a maintenance medium comprised of DMEM with Glutamax®, 10% foetal calf serum, 1% non-essential amino acids, 1% sodium pyruvate and 50 U/ml penicillin and 500 μg/ml streptomycin. Medium was replaced every second day for 6 days and daily thereafter until use at between day 20 and 30 when the cells were fully differentiated and gave a TER of 250–500 Ω cm^2.

For flux experiments, six well plates containing the monolayers on Snapwell filters were mounted on an orbital shaker at a speed of approximately 60 rpm and maintained throughout at 37°C. The apical (donor) buffer comprised 1 ml Hank's Balanced Salt Solution (HBSS) containing 11 mM D-glucose and 25 mM HEPES, pH 7.4. The basolateral (recipient) compartment contained 2 mls of the buffer. 200 μl samples were taken from the recipient side every 30 minutes over 120 minutes for anti-Factor Xa assay for heparin, HPLC for SNAC or scintillation counting for [3H]-SNAC (specific activity, 43 Ci/mmol). Samples were taken from the donor side at zero and 120 minutes in order to calculate mass balance. After each sample was taken, the filters containing the donor solution were removed and placed in fresh recipient buffer in order to maintain sink conditions. The heparin concentrations used on the apical side were 500 and 1000 I.U./ml (where 176 I.U. = 1 mg heparin).

Papp values were calculated using the following equation (16):

$$P_{app} = \frac{K \cdot V_{bl}}{A}$$

Where K = slope of Fa_{cum} against time, V_{bl} = volume on the basolateral side (2 mls), A = area of the monolayer (1.13 cm^2).

Fa_{cum} is defined as $\Sigma\ C_r/C_d$ in which C_r is the receiver side concentration for the time interval (seconds) and C_d is the donor concentration for the corresponding time interval.

Assays

HPLC of SNAC

SNAC was measured by HPLC analysis using a Shandon, Hypersil 5μ C18 column, 5.0 cm × 4.6mm, and a gradient method. Detection was by UV absorbance at 244 nm. The mobile phases consisted of A: 1N HCl, acetic acid, acetonitrile and water in respective volumes of 2.5:2:100:900, and B: acetic acid, acetonitrile and water in respective volumes of 3:700:900. The gradient used was an increase in B from 0–100% in 7 minutes at a flow rate of 2 ml/minute. 50μl samples and standards were prepared in HBSS: mobile phase B, 2:3 (v/v). The standard curve was linear with a range of 25–500 μg/ml SNAC. This assay was also applicable to salicylic acid, a potential metabolite of SNAC. The retention time was 6.5 minutes for SNAC and 4.5 minutes for salicylic acid.

[3H]-SNAC and [3H]-mannitol were assayed by liquid scintillation counting (Wallac, System 1409).

Anti-Factor Xa and APTT Assays

Anti-Factor Xa activity as an indicator of heparin bioactivity was measured by the Coatest® heparin colorimetric plasma assay with a sensitivity of 0.1–0.6 I.U./ml. Standards were run in HBSS instead of plasma in the case of Caco-2 fluxes. APTT measurements (17) for the rat intestinal instillations were made using either of two interchangeable methods: an automated APTT Biotrack 512 Coagulation Monitor or a BBL Fibrometer with standard APTT reagents.

Statistics

Statistical comparisons for *in situ* rat intestinal instillations were made using repeated measures analysis of variance (ANOVA) to test for the coincidence of the profiles for each pair-wise comparison. In Caco-2 monolayers comparisons were made using the unpaired Student's t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Flux of Heparin in the In Situ Rat Intestinal Instillation Models

Ileal and colonic instillation models were used to examine heparin fluxes in the presence of SNAC and/or PG. In the first set of experiments four groups of rats received ileal instillations of 3ml/kg of test solution. The solutions comprised (per ml): (a) 500 I.U. heparin in HBSS (b) 500 I.U. heparin and 10 mg SNAC in HBSS (c) 500 I.U. heparin in 5% aqueous PG and (d) 500 I.U. heparin and 10 mg SNAC in 5% aqueous PG. No heparin absorption was detected in any of these groups as indicated by APTT determination. Six rats were administered subcutaneous heparin as a positive control for heparin activity, each showing increases in APTT (not shown).

In the next set of intra-ileal experiments the volume of the test solution was reduced to 1 ml/kg (9) and increased doses of SNAC and heparin were administered. Heparin (1400 I.U) dosed in either HBSS or in 25% PG had no effect on APTT in any animal (Figure 1A, 1B). Significant increases in APTT with respect to heparin controls were evident upon dosing combinations of heparin and 35 mg SNAC in either HBSS or in 25% PG ($P < 0.05$ and $P < 0.001$ respectively). Furthermore, the magnitude of the APTT response to heparin was related to the concentration of SNAC, ie. 35 mg of the agent giving a significantly higher APTT than 17.5 mg ($P < 0.05$). Although SNAC (17.5 mg) with heparin did not statistically increase APTT over control values, solutions containing 17.5 mg of SNAC in 25% PG with heparin gave statistically increased elevations in APTT (Figure 1A, $P < 0.01$).

Results from the intra-colonic study (Figure 2), confirmed the main elements of the intra-ileal study and can be examined in comparison with Figure 1A. APTT was unaffected by administration of heparin (1400 I.U.) in HBSS or of heparin in PG (25%). SNAC (17.5 mg) administered with heparin was capable of statistically elevating APTT in the presence and absence of PG in comparison to the heparin controls ($P < 0.001$ in each case). PG alone had no effect on heparin absorption, but permitted a statistically larger APTT response to heparin in the presence of 17.5 mg SNAC compared to that seen with 17.5 mg SNAC in HBSS ($P < 0.01$). The APTT responses obtained following intra-colonic instillation of heparin with SNAC (Figure 2) gave a greater peak effect but were less transient than those of the corresponding intra-ileal instillations (Figure 1A).

Independent histological examination of the ileal tissues after 5 hours exposure to 35 mg SNAC, 25% PG and the combination of both with heparin (1400 I.U.), indicated no significant necrotic damage to the epithelium in response to any of the treatments in comparison to vehicle controls (Figure 3). Some very minor effects of PG, SNAC and the combination in 50% of the sections (n = 3–4) included occasional epithelial cell shedding, some villi fusion and congestion of mucosal capillaries with blood. Such findings are also common in sections from control animals and are attributable to focal trauma at surgery. Overall, the histology of the rat ileal mucosa indicated a high tolerance to SNAC and PG, with no evidence of significant intestinal toxicity *in situ*.

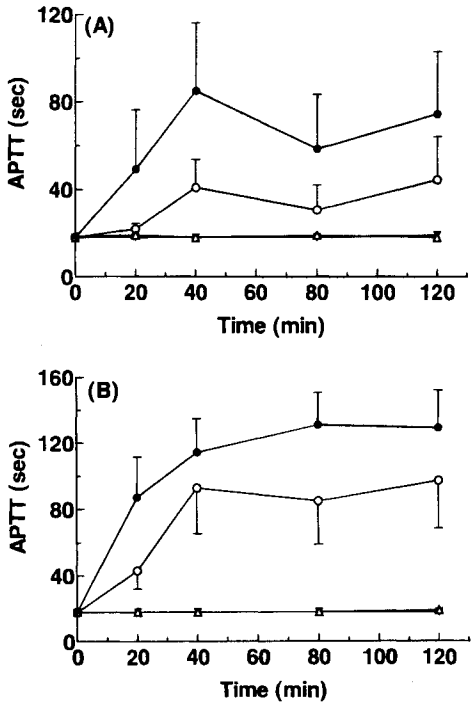

Fig. 1. Effects of SNAC and PG on APTT assay after intra-ileal administration in rats. The heparin dose throughout was 1400 I.U. (1 ml/kg). **A.** Heparin in HBSS (Δ), heparin in 25% PG (▼), heparin with 17.5 mg SNAC in HBSS (○), heparin with 17.5 mg SNAC in 25% PG (●). **B.** Heparin in HBSS (Δ), heparin in 25% PG (▼), heparin with 35 mg SNAC in HBSS (○) and heparin with 35 mg SNAC in 25% PG (●). Data are expressed as mean ± SEM for each time point (N = 6 in each group). Note the difference in scale on the y axis between A and B.

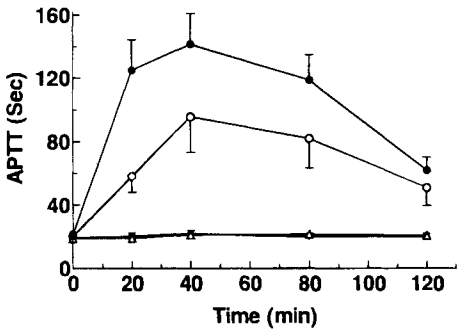

Fig. 2. Effects of SNAC and PG on APTT assay after intra-colonic administration in rats. The heparin dose throughout was 1400 I.U (1 ml/kg). Groupings were: heparin in HBSS (Δ), heparin in 25% PG (▼), heparin with 17.5 mg SNAC in HBSS (○), heparin with 17.5 mg SNAC in 25% PG (●). Data are expressed as mean ± SEM for each time point (N = 15 in each group).

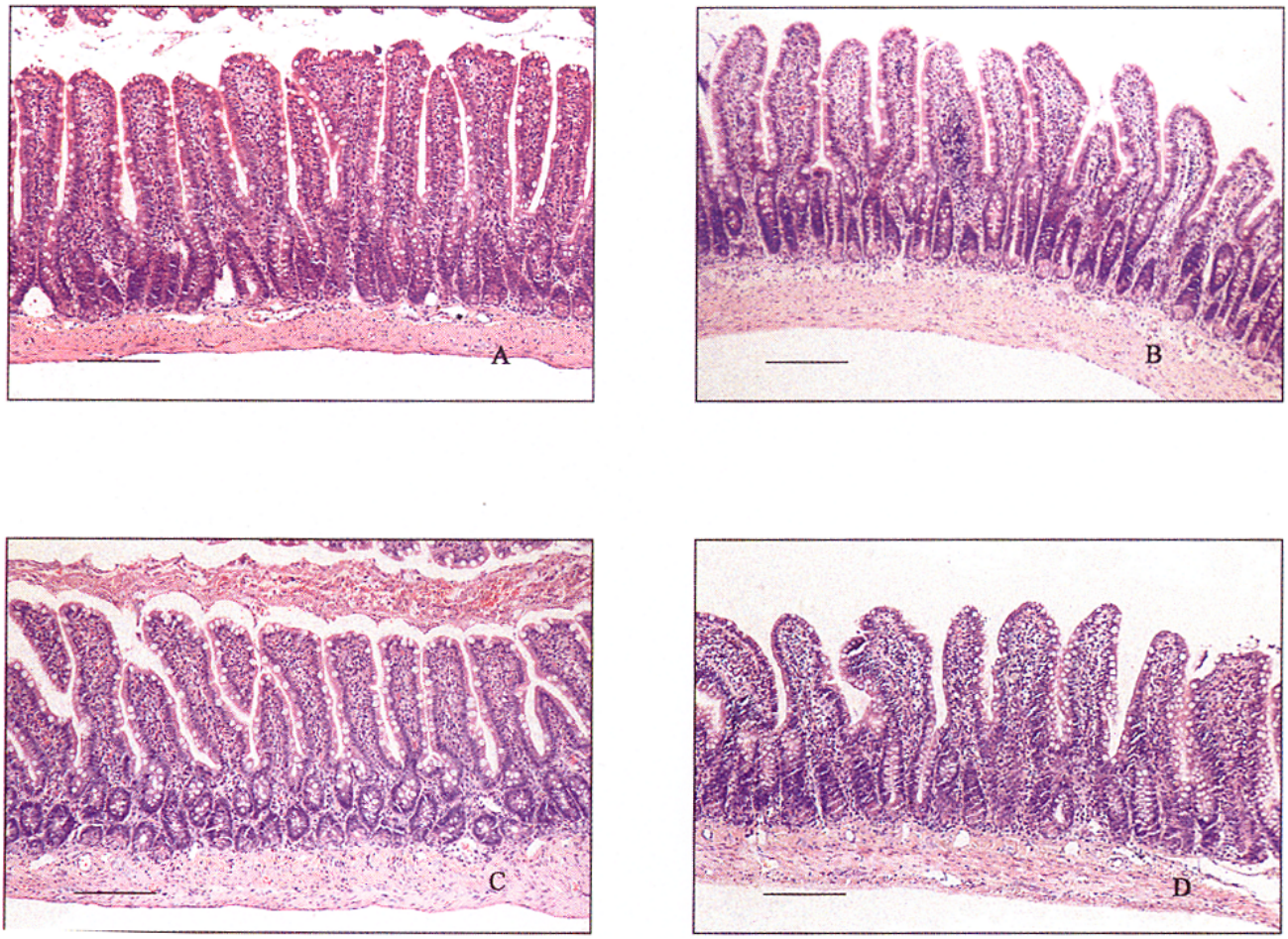

Fig. 3. H & E photomicrograph of rat ileal tissue sections after 5 hours exposure to SNAC and PG. **A.** HBSS vehicle control, **B.** 25% PG, **C.** 35 mg SNAC and **D.** 35 mg SNAC and 25% PG and 1400 I.U. heparin. Horizontal bars denote 125 μm on each micrograph.

Effects of SNAC and PG on Caco-2 Cell Viability: EM, TER, and [^{3}H]-Mannitol Fluxes

Figure 4 shows representative transmission electron micrographs (EM) of Caco-2 exposed to SNAC and/or PG. Untreated Caco-2 showed healthy tight junctions, microvilli, mitochondria and desmosomes after a 2 hour incubation in HBSS (Figure 4A). Neither 1% PG nor 10 mg/ml SNAC (Figure 4B, 4D) altered the appearance of the epithelium. Monolayers exposed to either 10% PG or to 25 mg/ml SNAC under identical conditions showed a disoriented cell layer with evidence of porosity and an increase in vacuolisation (Figure 4C and 4E respectively). In the presence of 10 mg/ml SNAC (Figure 4F), the cytotoxic effects of 10% PG were not apparent. Concentrations of heparin up to 1000 I.U./ml had no effect on cell appearance (results not shown).

The effects of SNAC and PG on TER values in Caco-2 were in agreement with the main EM observations. The results in Table 1 show that PG at concentrations of 5% and 10% reduced TER with respect to control monolayers after exposure on the apical side for 120 minutes. TER was also decreased in the presence of 25 and 50 mg/ml SNAC. In agreement with the EM, TER was maintained in the presence of 10 mg/ml SNAC and this concentration of SNAC also appeared to eliminate the decrease seen with 5% PG.

Considerable variability was observed in the [^{3}H]-mannitol fluxes across all of the groupings (Table 1). Apart from 10% PG or 50 mg/ml SNAC, all other groupings gave fluxes within the normal range confirming that limited decreases in TER are not necessarily mirrored by large changes in mannitol flux (18). 50 mg/ml SNAC (the concentration that was used *in situ*) obliterated TER and increased mannitol flux over 25 fold, effects consistent with a compromise in barrier properties. In neutral red cytoxicity experiments, the IC_{50} for SNAC in Caco-2 was 35 mg for a 2 hour exposure and, in addition, the monolayers could not recover TER after exposure and subsequent removal of 25 mg/ml SNAC (results not shown). Though PG at a concentration of 10% was cytotoxic, the monolayers recovered after exposure to 10% PG when placed back in culture medium, the only concentrations of these agents which did not compromise the epithelial barrier could be validly selected for heparin flux testing.

Flux of Heparin Across Caco-2

Following the addition of 500–1000 I.U. heparin/ml to the apical side of monolayers no heparin was detected basolaterally (Table 2). Considerable inter-monolayer variability in the heparin fluxes was observed in the presence of SNAC, PG, or SNAC with PG. Within these groups, the Papp ranged widely from

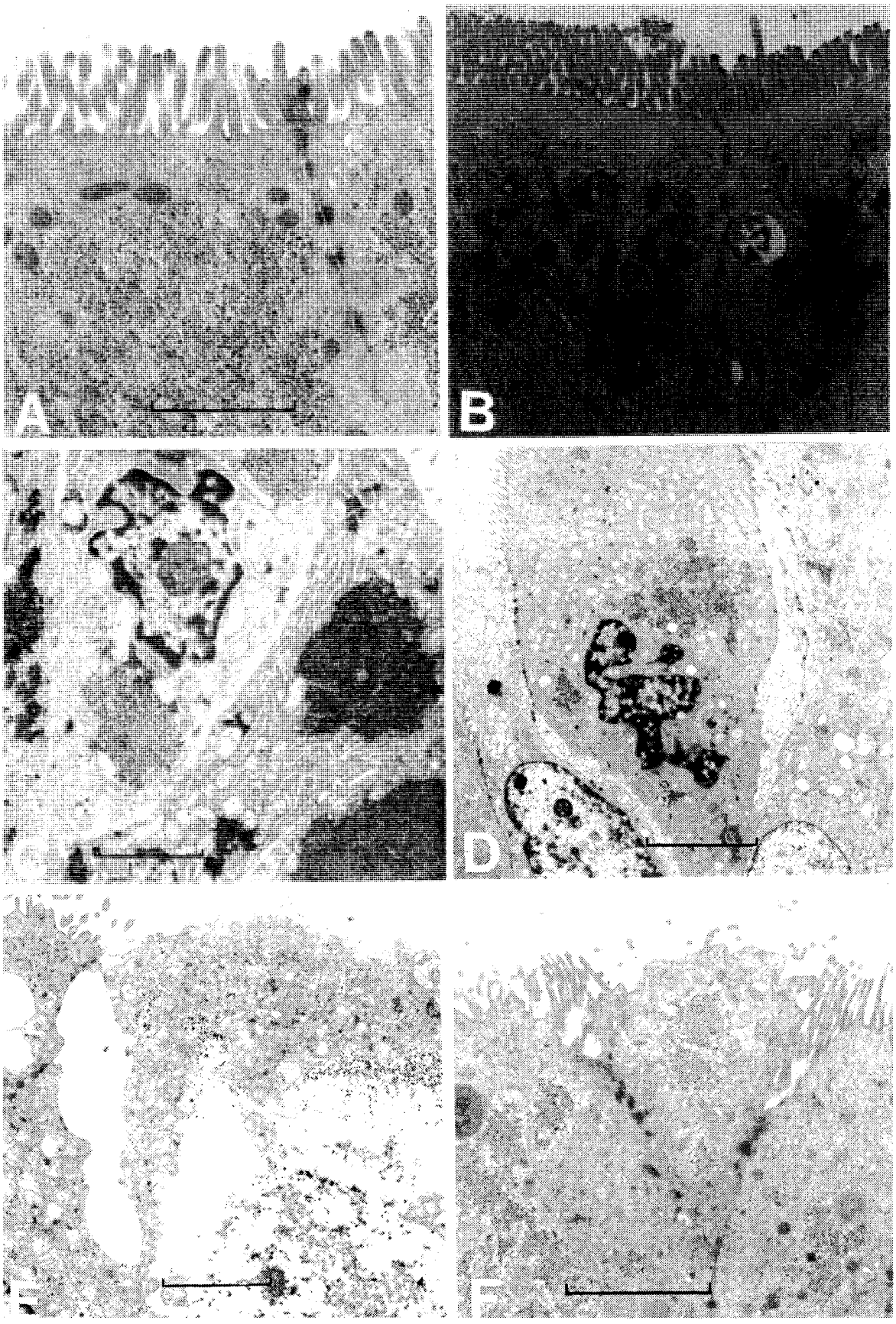
A
B
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Table 1. Effects of SNAC and PG on TER and [^{3}H]-Mannitol Fluxes in Caco-2 Monolayers

	%Control TER	Papp [^{3}H]-mannitol ($\times 10^{-6}$ cm/s)
Control	100.0	1.34 (0.31)
5% PG	43.7 (4.6)[a]	1.70 (0.63)
10% PG	28.5 (17.4)[a]	2.47 (0.74)[a]
10 mg/ml SNAC	122.5 (18.8)	1.94 (0.30)
25 mg/ml SNAC	61.0 (32.2)[a]	1.36 (0.24)
50 mg/ml SNAC	0[b]	36.43 (2.00)[b]
5% PG + mg/ml SNAC	94.0 (16.4)	1.00 (0.06)
10% PG + 10 mg/ml SNAC	59.2 (34.3)[a]	1.72 (0.53)

Note: TER values were normalised to a control value of 75.5 ± 9.5% remaining after 2 hours in HBSS.

[a] = $P < 0.05$. $P < 0.01$ (unpaired Student' t-test with respect to controls).

[b] N = 3–8 experiments in each group. Values are given as means with SD values given in brackets. 50 mg/SNAC obliterated TER in 30 minutes. The initial TER was 250–500 Ω cm^2.

Table 2. Heparin Absorption across Caco-2 Monolayers from the Apical to the Basolateral Side in the Presence of SNAC and/or PG as Detected Indirectly by Anti-Factor Xa

	SNAC (mg / ml)	PG (%)	Fluxes / Monolayers
CONTROL	—	—	0/14
SNAC	7–10	—	3/8
	50	—	4/4
PG	—	1–5	7/13
	—	10	5/5
SNAC/PG	7–10	1–5	4/8
	7–10	10	12/16

Note: Heparin concentrations on the donor side were 500 I.U./ml, except in the case of the controls where 500 or 1000 I.U./ml were used (n = 7 in each case). Results are expressed as the number of detectable fluxes per monolayers tested. The Papp for the flux across unseeded filters was 4.5×10^{-5} cm/s and the Papp range across those Caco-2 monolayers in which flux was detectable was 5.0×10^{-8} cm/s – 2.9×10^{-6} cm/s.

the limit of detection 5×10^{-8} cm/s to 2.9×10^{-6} cm/s, with a mean value of 2.0×10^{-7} cm/s. Table 2 shows that PG alone (1–10%) was capable of enhancing heparin flux across Caco-2 and that the effects of SNAC were neither reproducible nor discriminated from those of PG at the non-cytotoxic concentrations (10 mg/ml) tested. Only when monolayers were exposed apically to cytotoxic levels of SNAC (50 mg/ml), was the heparin flux increased reproducibly.

Flux of SNAC Across Caco-2

SNAC was well absorbed across Caco-2 monolayers as shown by a high absorptive mucosal-to-serosal Papp of 2.2×10^{-5} cm/s using donor concentrations of 1–10 mg/ml. The SNAC flux was linear over 2 hours and was of similar magnitude in the serosal-to-mucosal direction (Table 3). Filters without monolayers gave a significantly higher Papp of 10^{-4} cm/s, suggesting that the monolayer rather than the filter was the significant impediment to SNAC flux.

There was no evidence of salicylic acid formation during or after SNAC fluxes as determined by HPLC, indicating that SNAC was not metabolised. Despite a 1000 fold difference in donor concentration between labelled and unlabelled SNAC in separate experiments, the constant Papp values indicated unsaturable absorption (Table 3). Overall, the data indicated a passive and metabolically-stable transcellular absorption mechanism for SNAC across Caco-2.

The effects of heparin (500 I.U./ml) and PG (1%, 5% and 10%) on SNAC absorption across Caco-2 were determined. The presence of either PG and/or heparin statistically reduced the Papp of SNAC at each of the concentrations tested (Figure 5) A concentration-dependent reduction in the Papp for SNAC was observed in the presence of PG. Thus, 10% PG reduced the Papp for SNAC more than 5% PG or 1% PG ($P < 0.001$ for each comparison). The combination of PG (1%, 5% or 10%) with heparin caused a larger reduction in the Papp for SNAC than in the presence of PG alone ($P < 0.05$ for each comparison). Similarly, the combination of heparin with PG (1% or 10%) gave a larger reduction in the Papp for SNAC in comparison to that of heparin alone ($P < 0.05$ for each comparison). These results suggest that the combination of PG and heparin exerts an additive effect on the reduction of the Papp for SNAC across Caco-2.

DISCUSSION

Intestinal drug absorption models such as *in situ* rat intestinal instillations and Caco-2 monolayers can offer important mechanistic information for the design of formulations. The present study shows the importance of using several intestinal absorption models in parallel to minimize limitations of individual systems (19).

Rat *in situ* intra-ileal instillation results showed that SNAC increased heparin transport in the presence and absence of PG. Therapeutically, an increase in APTT of 1.5–2.5 times baseline is required to give anti-coagulant effects without the risk of internal bleeding (20). Such increases were detected within the range of 17.5–35.0 mgs SNAC in the ileal and colonic instillations. While PG had no direct effect on APTT in either

Table 3. Papp Values of SNAC across Caco-2 Monolayers

Condition	Papp of SNAC ($\times 10^{-6}$ cm/s)
Unseeded filters	115.0 (0.5)
Caco-2 (m - s)	22.3 (0.5)
Caco-2 (s - m)	19.7 (1.6)
Caco-2 ([^{3}H]-SNAC) (m - s)	24.0 (0.2)

Note: N=3–9 in all cases. SD values are given in brackets. m - s indicates flux from mucosal-to-serosal and s - m indicates serosal-to-mucosal flux. The starting concentrations of SNAC and [^{3}H]-SNAC were 1–10 mg/ml and 10 μg/ml respectively.

Fig. 4. *(Opposite)* TEM micrographs of the effects of SNAC and PG on Caco-2 monolayers after 2 hours exposure. **A.** Control Caco-2. **B.** 1% PG. **C.** 10% PG. **D.** 10 mg/ml SNAC. **E.** 25 mg/ml SNAC. **F.** 10 mg/ml SNAC and 10% PG. Horizontal bars denote 1 μm on each micrograph.

of the *in situ* rat models, the effects of SNAC on APTT were increased when PG was present. Relative to 250 I.U. subcutaneously administered heparin, the bioavailabilities for 1400 I.U. ileally-instilled heparin were calculated as 7.9% and 18.4% for 17.5 mgs and 35 mgs SNAC respectively, and 13.7% and 26.5% for the same doses with 25% PG. Importantly, the histological assessment of selected ileal loops after lengthy exposure to high concentrations of SNAC, PG or the combination, revealed no evidence of necrosis.

From a formulation perspective, optimisation of the concentrations of all three agents is required even though heparin bioavailability was demonstrated *in situ* using SNAC in the absence of PG. As PG is a non-toxic additive in a wide range of pharmaceutical and food preparations (21), low concentrations might be included in a final product in order to permit reduced concentrations of SNAC to be used. The pH-dependent solubility of SNAC is also important in the design of an oral formulation. All the *in situ* instillations were carried out at pH 7.4 where the maximum solubility of the sodium salt is approximately 160 mg/ml (unpublished results). At acid pH the maximum solubility of the sodium salt is 2.5 mg/ml, indicating that protection against precipitation in the stomach may be helpful. However, we cannot discount the possibility that precipitation of a bioactive colloid of SNAC in the stomach may ultimately produce material which is re-dissolved at the higher pH of the small intestine and is localised in high concentration with heparin at the brush border membrane. In support of maintaining the two agents in close contact in a low volume, ileal instillations containing rhGH with a delivery agent of the same class administered in 3ml/kg solutions produced far less effect on transport than those studies in which the same dose was administered in a volume of 1 ml/kg (9).

In Caco-2 monolayers no basal heparin flux was detected. This observation is consistent with the 0.2% flux per hour detected for [^{3}H]-Fragmin in Caco-2 (22). Reproducible enhanced heparin absorption was only detected in Caco-2 monolayers in the presence of cytotoxic concentrations of PG and SNAC. Moreover, the effects of SNAC on absorption could not be discriminated from those of PG in this model. The inconsistency in the Caco-2 heparin studies may be because heparin levels on the recipient side were at the lower limit of detection of the anti-Factor Xa assay. However, cumulative heparin flux experiments across Caco-2 did not give any more reproducible data (not shown). Possible sequestration of heparin by enterocytes could be a second factor. Previous studies demonstrated that the heparin concentration was 1000 times higher in endothelial cells than in plasma after intragastric administration to rats (23). It seems unlikely however that intestinal epithelia concentrate heparin in the same way as thoracic blood vessel endothelia since mass balance studies for heparin fluxes across Caco-2, in the presence of SNAC and PG, accounted for 83% of the total administered (unpublished results). Therefore, little evidence of intracellular accumulation of heparin was seen in intestinal epithelia.

The most likely explanation is that non-cytotoxic concentrations of SNAC (~10 mg/ml) were simply too low to permit adequate flux in Caco-2 since this was 5 times less than that which gave a measurable effect *in situ*. Yet enhanced heparin flux data seen with higher concentrations of SNAC (25–50 mgs/ml) was invalid because the barrier properties of the monolayers were compromised. Other compounds such as sodium caprate and polysorbates also display higher cytotoxic sensitivity in Caco-2 than in whole tissue models (24). This is possibly due to direct and prolonged access to the apical membrane *in vitro*. Protective effects of mucus and rapid cell turnover, allied to physiological factors such as transit time and dilution are all important factors in preventing excipient-induced membrane damage *in vivo* (25). Combination of these factors may indeed permit higher concentrations of such agents to be used *in vivo* than *in vitro*.

Taken together, the following evidence supports a passive transcellular mechanism for SNAC across Caco-2 epithelia: 1. Equal and unsaturable SNAC flux in the secretory and absorptive directions, 2. A high Papp value, unchanged over a wide range of concentrations and 3. A non-polar octanol/water coefficient of 0.7. From our curve of Papp values for passively absorbed reference compounds versus absorption in man (26), a Papp of 2.2×10^{-5} cm/s for SNAC corresponded to intestinal absorption of 80–90%.

It is possible that SNAC can be hydrolytically cleaved in the intestine to give salicylate or salicylamide. Sodium salicylate is known to promote insulin absorption *in vivo* (27) by reducing insulin aggregation (28). However, SNAC was not metabolised to salicylic acid derivatives during transport across Caco-2 monolayers as shown by HPLC analysis, so it is unlikely that such a metabolite is the active absorption promoter of heparin absorption under these conditions.

The reduced Papp for SNAC in the presence of PG and/or heparin indirectly supported the hypothesis (29) that this type of drug delivery agent may facilitate hydrophobic non-covalent interactions between the agent, the drug and the excipient. It is possible therefore that the concentration of free SNAC on the apical side of the monolayer is reduced by association with either or both of the other agents in solution. Direct heparin interaction with non-α-amino acids was also suggested from recent heparin/methylene blue dye displacement and heparin affinity chromatography experiments *in vitro* (10). It is unlikely that the reduced Papp for SNAC in the presence of PG and/or heparin was due to competition for a common receptor on the apical cell membrane since all three agents have different

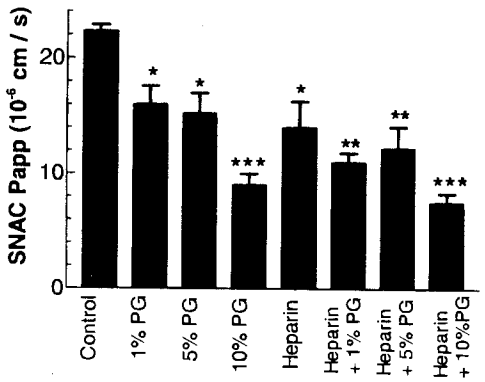

Fig. 5. Papp of SNAC across Caco-2 in the presence of PG and/ or heparin. In each case n = 4 − 9 experiments. Values are given as the mean ± SD. The SNAC concentration was 10 mg/ml and the heparin concentration was 500 I.U./ml. Each set of values was compared to control Papp of SNAC. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Further comparisons between individual groups are given in the text.

structures and do not undergo receptor-mediated transport. Diffusion of SNAC may also be reduced in the presence of high concentrations of each agent as a result of increased viscosity.

In conclusion, these studies demonstrate that administration of SNAC to the intestinal tract significantly increased heparin bioavailability. PG had a beneficial effect on SNAC-induced heparin absorption but its presence was not obligatory. The concentrations of SNAC which permitted heparin flux in rat *in situ* instillations did not damage the tissue in comparison with cytotoxic effects detected *in vitro,* concentrations below which reproducible enhancement of heparin absorption was not seen.

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