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Research Article

In Vitro Evaluation of Dexamethasone-β-D-Glucuronide for Colon-Specific Drug Delivery

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Dexamethasone-β-D-glucuronide is a potential prodrug for colonic delivery of the antiinflammatory corticosteroid dexamethasone. Previous studies [T. R. Tozer *et al.*, *Pharm. Res.* 8:445–454 (1991)] indicated that a glucoside prodrug of dexamethasone was susceptible to hydrolysis in the upper gastrointestinal tract. Resistance of dexamethasone-β-D-glucuronide to hydrolysis in the upper gastrointestinal tract was therefore assessed. Conventional, germfree, and colitic rats were used to examine enzyme levels along the gastrointestinal tract to compare the stability of two model substrates (*p*-nitrophenyl-β-D-glucoside and -β-D-glucuronide) and to evaluate the prodrug dexamethasone-β-D-glucuronide. Hydrolytic activity was examined in the luminal contents, mucosa, and underlying muscle/connective tissues in all three types of rats. Enzymatic activity (β-D-glucosidase and β-D-glucuronidase) was greatest in the lumen of cecum and colon of conventional rats. In contrast, germfree rats exhibited relatively high levels of β-D-glucosidase activity (about 80% of total activity in the conventional rats) in the proximal small intestine (PSI) and the distal small intestine (DSI). Rats with induced colitis (acetic acid) showed reduced levels of luminal β-D-glucuronidase activity in the large intestine; however, β-D-glucosidase activity was relatively unchanged relative to that of the conventional rat. Mucosal β-D-glucuronidase activity was significantly lower in the colitic rats compared with that in the conventional animals. Despite reduced luminal levels of β-D-glucuronidase activity in the colitic rats, there was still a sharp gradient of activity between the small and the large intestines. Permeability of the glucoside and glucuronide prodrugs of dexamethasone through a monolayer of Caco-2 cells was relatively low compared to that of dexamethasone. The results indicate that dexamethasone-β-D-glucuronide should be relatively stable and poorly absorbed in the upper gastrointestinal tract. Once the compound reaches the large intestine, it should be hydrolyzed to dexamethasone and glucuronic acid. Specificity of colonic delivery in humans should be even greater due to lower levels of β-D-glucuronidase activity in the small intestine compared with that in the laboratory rat.

KEY WORDS: colon-specific drug delivery; dexamethasone; dexamethasone-β-D-glucuronide; intestinal microflora-mediated drug hydrolysis; prodrug; inflammatory bowel disease.

INTRODUCTION

Colon-specific drug delivery offers the potential to improve the therapy of inflammatory bowel disease (IBD). At present, the treatment of IBD often involves systemic and topical (enema) administration of corticosteroids (1). To overcome the common side effects of corticosteroid therapy (muscle wasting, osteoporosis, growth retardation in children, "moon faces," hypertension, diabetes), topically active corticosteroids have been under investigation. Unless administered as an enema, most of the topically active corticosteroids, such as beclomethasone dipropionate, prednisolone metasulfobenzoate, tixocortol pivalate, and budesonide, are generally not effective in controlling inflammation in the colon. Therefore, a number of approaches are being developed to deliver these and other therapeutics locally to the mucosal epithelium of the large intestine via the oral route (2,3).

One approach to delivering drugs to the large intestine involves the use of prodrugs. Two clinically useful prodrugs are sulfasalazine and olsalazine (4). These prodrugs deliver 5-aminosalicylic acid to the colon to control inflammation following reduction of the azo bond present in these prodrugs. Delivery of corticosteroid prodrugs to the large intestine requires a different delivery mechanism than that used for 5-aminosalicylic acid. One possibility is to prepare glycoside derivatives of corticosteroids which possess a primary hydroxy group (normally found in the C-21 position). The glycoside prodrugs are larger and more hydrophilic than the drug. These properties tend to reduce the rate of absorption from the gastrointestinal tract. Once the prodrug reaches the large intestine, the polar glycoside residue should be hydrolyzed by glycosidases of colonic bacteria (5).

The use of glycosides in colonic drug delivery finds its

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basis in the disposition of dietary glycosides following ingestion. These glycosides include flavonoids, cyanogenic glycosides, amygdalin, cycasin, and many others. Bacterial hydrolysis of naturally occurring plant glycosides in the gut has been reviewed by Brown (6). The ability of any glycoside to carry to and subsequently release an aglycone in the large intestine depends on the prodrug's susceptibility to hydrolysis along the entire length of the alimentary canal. In addition, the glycoside should be poorly absorbed from the lumen of the gastrointestinal tract. Previous studies in the guinea pig (7) indicated that the glucoside prodrug of dexamethasone was less stable in the small intestine than desired. The data suggested that there was substantial β-glycosidase activity in the lumen of the small intestine derived from sloughed enterocytes (7). The present study was performed to examine the stability of another prodrug, dexamethasone-β-D-glucuronide, in various locations of the rat gastrointestinal tract (luminal contents, mucosa, and remaining gastrointestinal tissues—muscularis mucosa, submucosa, and smooth muscle) of the conventional rat, the germfree rat, and the colitic rat. Use of germfree rats permits estimation of the relative contribution bacterial and intestinal glycosidase activity in the lumen of the small and large intestine. Also, the relative rates of drug and prodrug permeation across a model colonic membrane (Caco-2 monolayers) were measured.

MATERIALS AND METHODS

Materials

Dexamethasone (U.S.P., micronized) was purchased from the Upjohn Co. (Kalamazoo, MI). Triamcinolone acetonide, *d*-equilenin, *p*-nitrophenyl-β-D-glucuronide, and β-glucuronidase (type IX from *Escherichia coli*) were purchased from Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenyl-β-D-glucoside was obtained from Fluka (Buchs, Switzerland). Dexamethasone-β-D-glucoside was prepared as described previously (8,9). Dulbecco's modified Eagle's medium (DMEM/High) with 25 m*M* Hepes and fetal bovine serum (Select) were purchased from JRH Biosciences (Lenexa, KS). MEM nonessential amino acid (100×) solution, L-glutamine (200 m*M*), penicillin–streptomycin solution, and trypsin–EDTA (0.05% trypsin, 0.53 m*M* EDTA) were from GIBCO Laboratories, Life Technologies Inc. (Grand Island, NY). Rattail collagen was bought from Collaborative Research Inc. (Bedford, MA). Cluster dishes with 12-mm Snapwells (0.4-μm pore size) and T-flasks were obtained from Costar (Cambridge, MA). The Caco-2 cell line (Batch No. F-9151) was from American Type Culture Collection (Rockville, MD). All the other chemicals and solvents were of high purity or analytical grade and were used as received. Elemental analysis was performed on a Perkin Elmer 2400 Elemental Analyzer.

Preparation of Dexamethasone-β-D-Glucuronide (9α-Fluoro-11β,17αdihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl β-D-Glucuronide)

Dexamethasone-β-D-glucuronide was prepared by a modified Koenigs–Knorr reaction. Dexamethasone (1.65 g, 4.2 mmol) was dissolved in dry chloroform (200 mL) over 4-Å molecular sieves in a 500-mL round bottom flask. After 10–20 mL had been distilled, freshly prepared Ag_2CO_3 (10) (5.4 g, 10.2 mmol) was added to the flask. Then a solution of 2,3,4-tri-*O*-acetyl-1-bromo-α-D-methyl glucuronic acid (4.8 g, 14.7 mmol) in dry $CHCl_3$ (100 mL) was added dropwise from an addition funnel. The reaction mixture was protected from light and stirred throughout. The addition of bromosugar took approximately 1 hr, and the solvent was distilled continuously during that time. Distillation was continued for an additional hour by the addition of dry $CHCl_3$ (100 mL). Then the solution was filtered, washed with cold saturated NaCl solution, and dried (Na_2SO_4) and the solvent removed. The oily residue was dissolved in several milliliters of MeOH/H_2O (65:35, v/v) and purified by flash column chromatography on RP-18 packing material (Baker Chemical Co., Phillipsburg, NJ). The appropriate fractions were collected, and the solvent was removed under reduced pressure. The product was crystallized from MeOH/H_2O to yield 1.60 g (53.6%) of dexamethasone acetyl methyl glucuronide. This product was characterized by IR and ^{1}H-NMR analyses. The protecting groups on the sugar were removed by treating dexamethasone acetyl methyl glucuronide (1.5 g; 2.1 mmol) with 0.2 *N* NaOH (100 mL) for 15 minutes, followed by the addition of H_2O (100 mL). After an additional 30 min, the solution was neutralized by the addition of dilute acetic acid in MeOH. The solvent was then removed under reduced pressure and the resulting oil was dissolved in several milliliters of MeOH/H_2O (50:50, v/v) followed by purification on the RP-18 flash column (eluant:MeOH/water, 50/50, v/v). The product, dexamethasone-β-D-glucuronide, was collected, the solvent was removed under reduced pressure, and the resulting oil was dissolved in 10–15 mL pure H_2O. The solution was frozen and the water removed by lyophilization to yield 0.99 g (80%). The structure of the glucuronide prodrug was confirmed by ^{1}H-NMR and IR spectroscopy: ^{1}H NMR: δ (ppm) 0.87 (s, 3H, C-18), 3.31 (s, 5H, OH), 4.28 (d, 1H, C-1′, J = 7.2 Hz), 4.49 (AB q, 2H C-21, J = 18.3 Hz), 6.00 (d, 1H, C-2, J = 7.2 Hz), 7.28 (d, 1H, C-1, J = 18.3 Hz). IR (KBr): 3450 (OH), 1720 (C=O), 1660 (C=C), 1600 (COO^-). Analytical calculated for $C_{28}H_{35}O_{11}FNa$: C—calcd 56.95, found 55.99; H—calcd 5.93, found 5.73.

Animals

For the *in vitro* hydrolysis studies gastrointestinal (GI) tracts of three groups of male Sprague Dawley rats (200–270 g, 6 to 7 weeks old) were used. Conventional rats were purchased from Simonsen Labs (Gilroy, CA) and housed in the animal care facility at SRI International. A uniform pancolitis was induced to the second group of rats (colitic rats) by flushing 4% acetic acid through the cecal-ascending junction and out the rectum (11). The colitis was induced at the Division of Gastroenterology of the University of Alberta (Edmonton, Canada) and the rats were sacrificed 48 hr after induction of the colitis. Germfree rats were obtained from the Gnotobiote Laboratory of the University of Wisconsin (Madison). All rats had free access to food (Purina certified chow) and water until they were sacrificed by carbon dioxide anesthesia. Immediately after death of the animal, the carcass was opened by bilateral thoracotomy and the GI tract was isolated. Following removal of fatty and mesenteric tis-

sues, the GI tract was segmented into stomach, small intestine, cecum, and colon. The small intestine was further divided into proximal and distal segments of equal length. Then the whole GI tract segments were immediately quick-frozen and stored at −30°C until use. Previous experiments had shown that freezing of GI tract parts does not alter enzyme activity of the samples if they are kept at −30°C for 1 to 2 months and analyzed upon thawing.

In Vitro Hydrolysis Studies

Preparation of Homogenates. The rates of hydrolysis of *p*-nitrophenyl-β-D-glucoside (*p*-NP-glc), *p*-nitrophenyl-β-D-glucuronide (*p*-NP-glrd), and dexamethasone-β-D-glucuronide were measured in the homogenized contents and tissues (mucosal scrapings and remaining muscle/connective tissues) of rat stomach, proximal small intestine (PSI), distal small intestine (DSI), cecum, and colon. For preparation of homogenates of the luminal contents, freshly isolated or thawed GI tract segments were rinsed with chilled 0.9% NaCl and gently squeezed out with wet tweezers to remove the luminal contents. Small intestinal contents were diluted to 10–20% slurries, and cecal and colonic contents to 5–10% slurries with 0.9% NaCl, respectively. The suspended contents were then homogenized under ice-cooling with an Ultra-Turrax homogenizer (type TP18/10SI, 20,000 rpm, Janke & Kunkel, IKA Werk, Staufen, Germany), followed by centrifugation at 500*g* (10 min at 4°C) to remove particulate material. Homogenization of mucosal scrapings should release both brush border and cytosolic enzymes. The resulting supernatants were kept on ice and used for incubations within 30 min. To prepare homogenates of mucosal scrapings, the washed intestines were cut open longitudinally and placed on a cold surface and the mucosa was scraped off with the edge of a microscope slide. The mucosal scrapings were diluted to 8–10 mL with 0.9% NaCl and homogenized for 30 sec (Ultra-Turrax). The remaining GI tissues (muscle layers and connective tissues) were cut into small pieces, diluted with 20 mL chilled 0.9% NaCl, and homogenized for 30 sec by a Kinematica Polytron (type PCU 1, speed 6–7; Brinkman Instruments, Westbury, NJ). After centrifugation of mucosal and tissue homogenates (500*g*/10 min/4°C), the fatty layer floating on the top of the samples was discarded and the supernatants were used for the incubations.

The protein content of the homogenates was determined by the Lowry technique (12) using standard solutions of bovine serum albumin for the calibration curve.

Hydrolysis Conditions. Incubations were performed at pH 6.5 for all homogenates and substrates, as well as at pH 4.5 for incubation of glucuronide substrates with homogenates of GI mucosa and tissues. The higher pH is close to the pH optima for hydrolysis by bacterial β-glycosidase from rodent and human sources (13) and close to the average pH in cecum/colon, whereas the pH 4.5 is close to the pH optimum for lysosomal β-glucuronidase activity (14). The pH of the incubation mixtures was adjusted with 0.1 *M* sodium phosphate buffer, pH 6.5, or 0.1 *M* sodium acetate buffer, pH 4.5. The incubations were run at 37°C in a shaking water bath. For the incubations of the model substrates the homogenates (3.0 mL of stomach and PSI contents, 2.0 mL of DSI contents, 1.0 mL of cecal and colonic contents, and 4.0 mL of mucosa and tissues, respectively) were diluted with buffer to a volume of 9.0 mL and preincubated for 5 min at 37°C. The reaction was started by adding 1.0 mL of a substrate stock solution (20 m*M* *p*-NP-glc or *p*-NP-glrd in 0.9% NaCl) to give an initial substrate concentration of 2 m*M*. Aliquots (1.6 mL) of the incubation were taken at 0 time and after incubation periods of 15 to 20 min at 37°C and the reaction was stopped by adding 0.5 *N* trichloroacetic acid (0.8 mL) to the samples. After centrifugation of the stopped reaction, 10 *N* KOH (0.1 mL) was mixed with 2.0 mL of supernatant and the amount of released *p*-nitrophenol was measured spectrophotometrically at 403 nm. The hydrolysis of dexamethasone-β-D-glucuronide was measured in the same manner except that a lower substrate concentration was used and the incubations were performed for 60 min (37°C). The homogenates (0.75 mL of small intestinal contents, 0.25 mL of large intestinal contents, and 1.0 mL of mucosa or tissue homogenate, respectively) were mixed with buffer to give a volume of 2.25 mL. After preincubation (5 min/37°C) a 5 m*M* aqueous dexamethasone-β-D-glucuronide solution (0.25 mL) was added, resulting in a substrate concentration of 0.5 m*M*. Aliquots (0.5 mL) of the incubation mixture were withdrawn at 0 time and after 60 min. The samples were added to chilled tubes containing 1.0 mL of saturated aqueous sodium chloride and internal standard and examined for extent of hydrolysis by high-performance liquid chromatography (HPLC) after extraction as described below. Blanks for all of the incubations were obtained by mixing the corresponding amounts of homogenate with buffer. Preliminary experiments with varying substrate concentrations had been performed to estimate the parameters V_{max} and K_m of the enzyme-catalyzed hydrolytic reactions. The final incubation concentrations of model substrates (2.0 m*M*) and dexamethasone-β-D-glucuronide (0.5 m*M*), respectively, were chosen to be at least 5–10 times higher than the corresponding K_m. Therefore, the incubations were performed under saturated conditions to obtain comparative constant velocity data (i.e., V_{max}) for the reactions in the different homogenates investigated. The hydrolytic activity present in the different homogenates was expressed as specific activity, i.e., nanomoles *p*-nitrophenol or dexamethasone released per milligram protein and minute (nmol × mg^{-1} × min^{-1}), and was calculated by dividing the amount of released *p*-nitrophenol or dexamethasone through protein content of incubation mixture and time of incubation. To assess the chemical stability of prodrug, control incubations in buffered solutions at pH 4.5 and 6.5, respectively (for at least 60 min at 37°C), were also performed.

Determination of Permeability Coefficients

Cell Culture. Cells were grown as described previously (15,16). Briefly, Caco-2 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM/High) with 25 m*M* Hepes, supplemented with 10% fetal bovine serum, 1% MEM nonessential amino acids, 1% L-glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 and 90% relative humidity. Cells grown in 75-cm^2 flasks were passaged every week at a split ratio of 1:3. Cells were trypsinized (10 mL, 5–10 min at 37°C) and

seeded at a density of 63,000 cells/cm^2 on prewetted (PBS; 15 min outside and then inside) collagen-coated Transwells polycarbonate filters, which were coated as follows: 1 part rattail collagen was mixed with 3 parts 60% ethanol. A 100-μL aliquot of this mixture was added to each insert, then dried for 4 hr in the hood with the lid slightly open. The medium was changed every other day. The monolayers were used between passage 26 and passage 31, and day 20 and day 30.

Permeability Measurements. Compounds were added to freshly prepared oxygenated (95% O_2/5% CO_2) Kreb's Ringer bicarbonate buffer (pH 7.4) supplemented with 40 m*M* D-glucose. The following amounts were used: 1.4×10^{-4} *M* dexamethasone, 9.0×10^{-4} *M* dexamethasone-glucoside, and 4.0×10^{-4} *M* dexamethasone-glucuronide.

Caco-2 monolayers growing on inserts (1.13 cm^2; 0.4-μm pore size) were washed free of media with Kreb's Ringer bicarbonate buffer prior to placement into an acrylic half-cell (Precision Instrument Design). The matching half-cell was joined to seal the diffusion apparatus and the chambers were then immediately placed in an aluminum block heater. Mucosal (donor) and serosal (receptor) solutions, 5.0 mL each, were placed into their respective chambers. Solutions were circulated by gas lift (95% O_2/5% CO_2). Concentration in the donor phase was determined at the beginning and at the conclusion of the experiment. These experiments were performed under sink conditions. Serosal samples (1.0 mL) were taken at appropriate time points, with replacement of the sampled volume. Samples were placed in vials, quick-frozen on dry ice, and stored at −20°C until analysis. All experiments were conducted at 37°C and continued for 2 hr. The permeability (*P*) was calculated according to the following equation:

$$P = \frac{V \cdot (dC/dt)}{A \cdot C_0} \quad (1)$$

where $V \cdot (dC/dt)$ is the change in mass as micrograms per unit time in the receiver chamber, A is the surface area of the monolayers, and C_0 is the starting concentration of the donor chamber.

Determination of *n*-Octanol/Water Partition Coefficients

The octanol/aqueous buffer (0.05 *M* phosphate, pH 7.4) partition coefficients (*K*) of dexamethasone, dexamethasone-β-D-glucoside, and dexamethasone-β-D-glucuronide were measured as described previously (9) with the following changes. Both phases were agitated overnight at room temperature. The initial concentration of the glucuronide was 0.1 mg/mL, dissolved in buffer.

Analytical Methods

Prodrug and dexamethasone levels were determined by reversed-phase HPLC. The chromatograph consisted of the following instruments (all from Waters Associates, Inc., Milford, MA): high-pressure pumps (Model 6000A), automatic sample injector (WISP 710B), UV-absorption detector (Model 481), and chromatography data station (Model 840). The separation was performed on a Whatman Partisil ODS-3 column (10 μm, 3.9 × 300 mm). A mobile-phase flow rate of 1.0 mL/min was used and the eluant was monitored at 246 nm. The mobile phase consisted of 0.02 *M* sodium acetate buffer (pH 4.8)/acetonitrile (68:32, v/v). All separations were performed at ambient temperature. The retention times observed under these conditions were 7.5 min (dexamethasone-β-D-glucoside), 14.5 min (dexamethasone), 18 min (triamcinolone acetonide), and 35 min (*d*-equilenin), respectively. Dexamethasone-β-D-glucuronide eluted with the solvent front. Calibration curves were obtained either by direct injection (15–30 μL) of ethanolic dexamethasone and dexamethasone-β-D-glucoside solutions (concentration of external standards, 5–100 μg/mL) or by injections of mixtures of varying amounts of dexamethasone with internal standards. Briefly, ethanolic solutions of dexamethasone (4 to 40 μg/mL) and the internal standards triamcinolone acetonide and *d*-equilenin (40 μg/mL each) were prepared. These solutions were combined (25 to 100 μL of dexamethasone solution, 25 μL of the internal standards each), evaporated by dryness under argon, and reconstituted in 120 μL of acetonitrile 50%. After vortexing, 80 μL was injected onto the HPLC column. The calibration curve prepared in the range of 100 to 4000 ng dexamethasone (amount of drug in 120 μL reconstituted sample) showed linearity ($r \geq 0.999$) with respect to ratios (dexamethasone/internal standard) of peak areas and peak heights. In general, the ratios of the peak areas were used for the calculations. The linearity of the detector response was tested up to a maximal amount of 80 μg of dexamethasone on the column.

All homogenates were treated identically to prepare the HPLC samples. After mixing of 0.5 mL of the incubation mixture with 1.0 mL of saturated saline containing 25 μL (40 μg/ml) of internal standard EtOH (*d*-equilenin for luminal contents, triamcinolone acetonide for mucosal scrapings and tissues, respectively), 4.0 mL of methyl-t-butyl ether/pentane (6:4, v/v) was added and each tube was vortexed for 5–10 sec, followed by centrifugation (5 min). The organic phase was transferred to another test tube, and the aqueous phase extracted once again by the same procedure. The combined organic extracts were transferred to an Eppendorf microfuge tube and evaporated to dryness under argon. The residue was reconstituted n 120 μL acetonitrile/water (1:1, v/v) and vortexed. After spinning on an Eppendorf centrifuge (Model 5412), 80 μL of the supernatants were injected into the HPLC. As chromatograms of extracts of luminal contents might have interfering peaks at the retention time expected for triamcinolone acetonide, the later-eluting *d*-equilenin was chosen as internal standard for these samples. Preliminary extractions of 0.9% NaCl and homogenates spiked with dexamethasone and internal standards resulted in recovery values of at least 95% for all three substances. Therefore, we did not perform the whole extraction procedure for the generation of the calibration samples.

To measure the amount of dexamethasone-β-D-glucuronide in the samples, a portion (0.5 to 1.0 mL) of the aqueous sample was incubated overnight (37°C) with 50 μL of β-glucuronidase solution (1 mg/mL in water). The sample was then extracted as described above and the amount of glucuronide was determined by the difference of β-glucuronidase-treated and untreated samples. Preliminary experiments showed that dexamethasone-β-D-glucuronide (sub-

strate concentration 0.01 m*M*) was quantitatively hydrolyzed within 8 hr under these conditions.

Dexamethasone-β-D-glucoside concentrations in aqueous samples were estimated without organic extraction. Prior to analysis, aliquots of dexamethasone-β-D-glucoside samples were either diluted or concentrated to prodrug levels within the range of the dexamethasone-β-D-glucoside calibration curve and injected directly into the HPLC.

Statistical Analysis

Results are presented as means ± SD (*in vitro* hydrolysis studies) or means ± SE (permeability measurements). Unless otherwise noted, statistical comparisons were made with analysis of variance (ANOVA) followed by Student–Newman–Keuls test for multiple comparison at a 95% confidence level. In the case of the permeability measurements ANOVA in combination with Fisher's Protected Least Significant Difference (PLSD) was used as the test statistics.

RESULTS

The *in vitro* hydrolysis studies with dexamethasone-β-D-glucuronide and the two *p*-nitrophenol model substrates in homogenates from various locations of the rat GI tract were designed to address the role of bacteria and host in colonic drug delivery using glycoside prodrugs. Figure 1 shows the specific enzymatic activity for hydrolysis of *p*-NP-glc and *p*-NP-glrd in homogenates of the luminal contents from stomach, PSI, DSI, cecum, and colon of conventional, colitic, and germfree rats, respectively. The highest hydrolytic activity was observed in the cecal and colonic contents of the conventional rat, whereas the contents of stomach, PSI, and DSI had much lower activity. The PSI had a significantly higher level of β-D-glucosidase activity compared with β-D-glucuronidase activity ($P < 0.05$, ANOVA), while the levels were comparable in the DSI. There was a 30-fold increase in specific activity of luminal β-D-glucuronidase between DSI and cecum in the conventional rats; the gradient of β-D-glucosidase activity between DSI and cecum was less pronounced. As shown in Fig. 2, the same general pattern of glycosidase activity was observed in the acetic acid-induced colitis model. In conventional rats, luminal β-D-glucosidase activity was slightly higher in the PSI than the DSI, while β-D-glucuronidase activity increased from the PSI to the DSI. However, the specific β-D-glucosidase activity in the two small intestinal segments was significantly lower than in the conventional rats ($P < 0.05$, *t* test), whereas cecal and colonic β-D-glucosidase activity was not significantly altered in the colitic animals. In contrast, the activities of the β-D-glucuronidase were higher in DSI and significantly lower in the large intestines of the colitic rats (cecum, $P < 0.05$; colon, $P < 0.01$, *t* test). The relative specific activity of the two enzymes in the lumninal contents of germfree rats is shown in Fig. 3. The levels were lower in all intestinal segments of the germfree rats compared with conventional rats. There was a higher level of β-D-glucosidase activity in the lumen of PSI and DSI compared with luminal β-D-glucuronidase activity. Luminal β-D-glucuronidase activity was very low in all GI tract segments of the germfree animals.

Enzyme levels in the mucosa of the GI tract of conventional, colitic, and germfree rats were also examined using the model substrates (see Figs. 4A–C). Mucosal β-D-glucosidase activity was measured at pH 6.5; β-D-glucuronidase activity, at pH 4.5 and 6.5. Highest β-D-glucosidase activity was observed in mucosal scrapings from the PSI of all animals. In addition, mucosal β-D-glucosidase activity of the germfree rat (Fig. 4C) showed the same general pattern as observed in the intestinal contents of germfree rats. The specific hydrolytic activity decreased progressively from PSI to cecum ($P < 0.05$, ANOVA). Mucosal β-D-glucuronidase showed significantly higher activity at the lower pH in all of the groups investigated. Mucosal scrapings from conventional and germfree rats showed greater β-D-glucuronidase activity in the large intestine than in the small

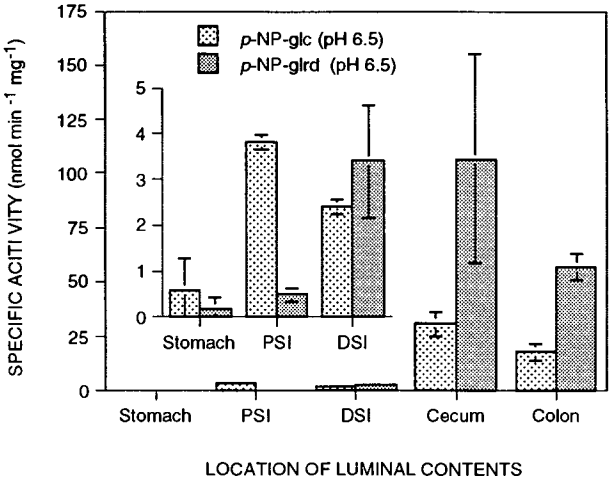

Fig. 1. Specific enzymatic activity (nmol mg^{-1} min^{-1}) for hydrolysis of *p*-nitrophenyl-β-D-glucoside (*p*-NP-glc) and *p*-nitrophenyl-β-D-glucuronide (*p*-NP-glrd) (substrate concentration, 2.0 m*M;* pH adjusted to 6.5 with 0.1 *M* sodium phosphate) in homogenates of the luminal contents from various GI tract locations of the conventional rat. Error bars are SD ($n = 3$).

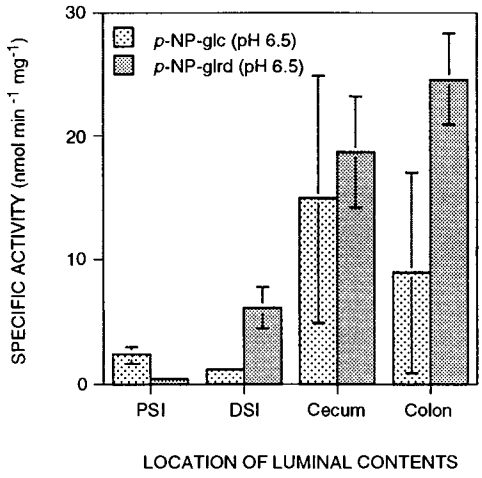

Fig. 2. Specific enzymatic activity (nmol mg^{-1} min^{-1}) for hydrolysis of *p*-nitrophenyl-β-D-glucoside (*p*-NP-glc) and *p*-nitrophenyl-β-D-glucuronide (*p*-NP-glrd) (substrate concentration, 2.0 m*M;* pH adjusted to 6.5 with 0.1 *M* sodium phosphate) in homogenates of the luminal contents from various GI tract locations of the colitic rat. Error bars are SD ($n = 3$).

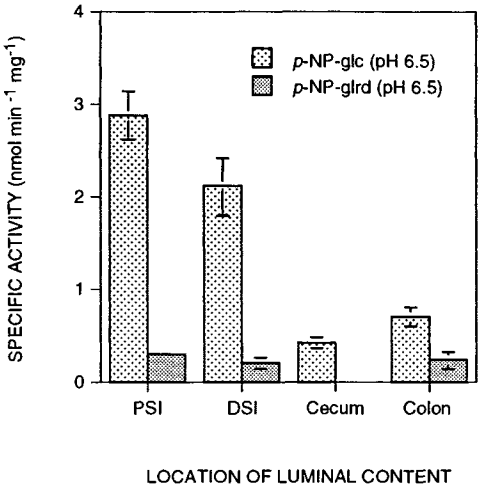

Fig. 3. Specific enzymatic activity (nmol mg^{-1} min^{-1}) for hydrolysis of *p*-nitrophenyl-β-D-glucoside (*p*-NP-glc) and *p*-nitrophenyl-β-D-glucuronide (*p*-NP-glrd) (substrate concentration, 2.0 m*M*; pH adjusted to 6.5 with 0.1 *M* sodium phosphate) in homogenates of the luminal contents from various GI tract locations of the germfree rat. Error bars are SD (n = 3).

intestine. This finding is consistent with that of Hsu and Tappel (17,18). In the colitis-induced rats (Fig. 4B), mucosal glycosidase activity varied only slightly along the entire length of the GI tract. Mucosal scrapings from the cecum and colon of the colitic rats showed significantly lower β-D-glucuronidase activity compound with that of conventional rats. Danovitch *et al.* (19) observed a similar difference in human colonic mucosa from patients with ulcerative colitis and control individuals. Germfree rats (Fig. 4C) showed significantly lower β-D-glucuronidase activity at pH 4.5 in the PSI than did both conventional and colitic rats.

Homogenates of the GI muscle tissues (data not shown) exhibited lower activity of β-D-glucosidase than the corresponding mucosal layers. There was no significant difference in muscle tissue β-D-glucuronidase activity among the conventional, the colitic, or the germfree animals at any site examined along the GI tract.

The hydrolysis of the prodrug dexamethasone-β-D-glucuronide was studied in a manner similar to that for model substrates. The observed distribution of hydrolytic activity in the luminal contents (Fig. 5) was that generally expected based on the previous experiments with the model substrate *p*-NP-glrd. The highest activity was measured in the cecal and colonic contents of the conventional animals, whereas the contents of PSI and DSI had much lower activity. There was a significant, 20- to 80-fold, increase in specific activity of luminal β-D-glucuronidase between DSI and cecum ($P < 0.05$, t test) in the conventional and colitic rats, respectively. A steady increase in luminal hydrolytic activity could be observed in both groups from the PSI to the cecum; however, these differences were statistically significant only between small and large intestine, and not within the two parts of the small intestine (PSI vs DSI) or the large intestine (cecum vs colon). The hydrolytic activity in the luminal contents of colitic rats was slightly higher in the DSI and slightly lower in the cecum compared to the conventional animals. However, direct statistical comparison (t test) of the corresponding GI tract segments did not reveal any significant changes in the luminal hydrolytic activity after induction of colitis. Also shown in Fig. 5 is the specific activity for hydrolysis of the dexamethasone-β-D-glucuronide in the luminal contents of germfree rats. In the lumen of the PSI, the activity was nearly identical to that of the conventional rat;

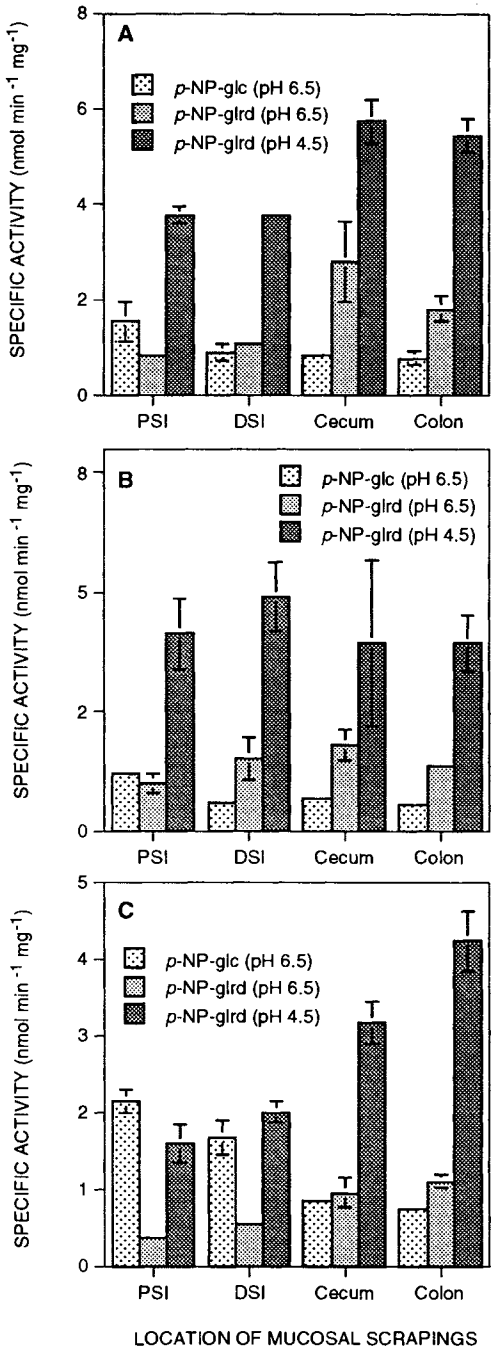

Fig. 4. Specific enzymatic activity (nmol mg^{-1} min^{-1}) for hydrolysis of *p*-nitrophenyl-β-D-glucoside (*p*-NP-glc) and *p*-nitrophenyl-β-D-glucuronide (*p*-NP-glrd) (substrate concentration, 2.0 m*M*; pH adjusted to 4.5 with 0.1 *M* sodium acetate or to 6.5 with 0.1 *M* sodium phosphate) in homogenates of the mucosal scrapings from various GI tract locations of the conventional rat (A), colitic rat (B), and germfree rat (C). Error bars are SD (n = 3).

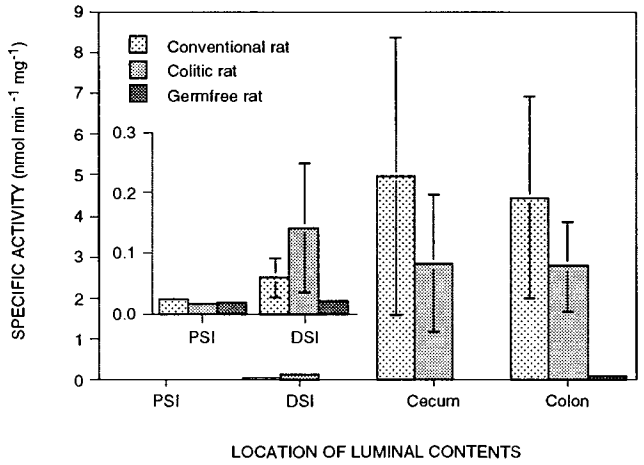

Fig. 5. Specific enzymatic activity (nmol mg^{-1} min^{-1}) for hydrolysis of dexamethasone-β-D-glucuronide (substrate concentration, 0.5 m*M;* pH adjusted to 6.5 with 0.1 *M* sodium phosphate) in homogenates of the luminal contents from various GI tract locations of the conventional, colitic, and germfree rat. Error bars are SD (n = 3).

in the DSI, the specific enzymatic activity from the germfree rats was about half that found in the DSI of conventional rats. As expected, there was very little activity in the lumen of the cecum and colon of the germfree rats compared with that of the conventional and colitic rats due to the absence of gut microflora and their associated enzymes.

Figure 6 shows the specific activities for hydrolysis of dexamethasone-β-D-glucuronide in mucosal scrapings from the conventional, colitic, and germfree rats. In general, mucosal hydrolytic activities in the small intestines were about two- to threefold higher at pH 4.5 than at pH 6.5 (data not shown). This finding suggests that mucosal β-D-glucuronidase activity is lysosomal in origin. Hydrolytic activity in the cecal musosa at pH 4.5 was significantly higher than in mucosal scrapings of PSI, DSI, and colon of colitic rats or mucosal scrapings of PSI and DSI of conventional rats, respectively ($P < 0.05$, ANOVA). No significant difference between the two groups was found when the hydrolytic activities in mucosal scrapings from the same intestinal location were compared. This finding was in contrast to the results reported with the model substrate *p*-NP-glrd, where a slight decrease in the β-D-glucuronidase activity in the mucosa of inflamed GI tract regions was observed.

The specific enzyme activity data were normalized to the amount of protein determined in the incubation medium. The amount of total protein present in the homogenates from the luminal contents and mucosal scrapings from the conventional, colitic, and germfree rats is shown in Fig. 7. In homogenates from the luminal contents of conventional rats, the protein content was relatively constant along the GI tract, although the levels were slightly higher in the large intestine. Protein contents of homogenates of colitic rats were approximately equal in the PSI, DSI, and cecum; there was a drop in total protein in the colon of the colitic rats. Protein content in homogenates of the luminal contents of germfree rats was relatively constant along the GI tract with the exception of the cecum, which had about four times the total protein of any other GI segment. The mucosal scraping homogenates showed similar profiles, regardless of rat type

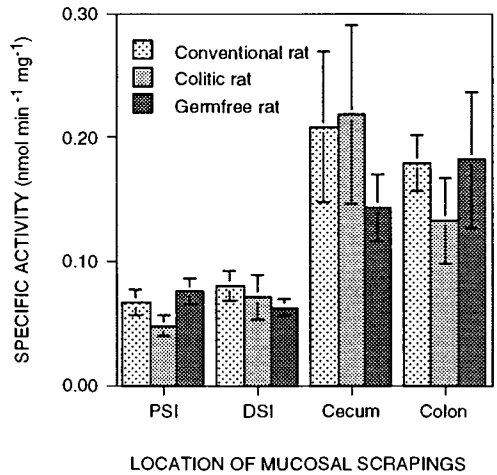

Fig. 6. Specific enzymatic activity (nmol mg^{-1} min^{-1}) for hydrolysis of dexamethasone-β-D-glucuronide (substrate concentration, 0.5 n*M;* pH adjusted to 4.5 with 0.1 *M* sodium acetate) in homogenates of the mucosal scrapings of various GI tract locations of the conventional, colitic, and germfree rat. Error bars are SD (n = 3).

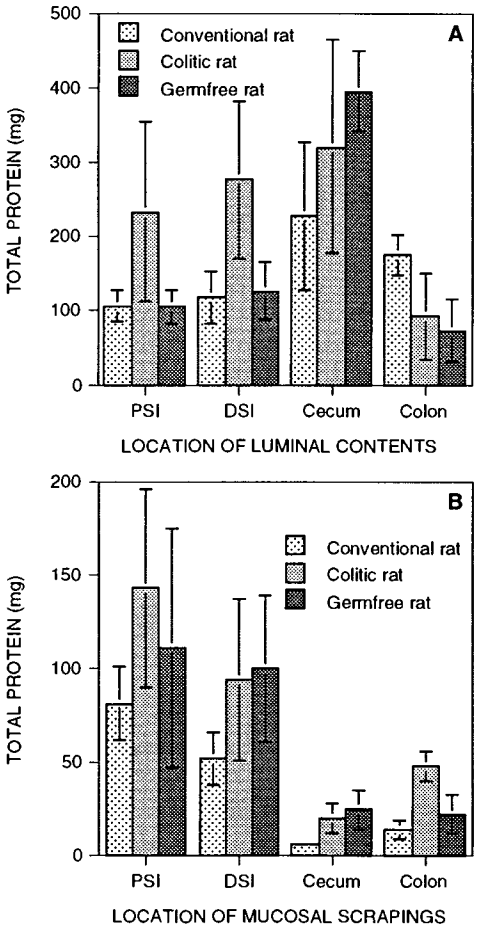

Fig. 7. Total protein measured in homogenates prepared from luminal contents (A) and mucosal scrapings (B) from various locations along the GI tract of conventional, colitic, and germfree rats. Total protein was determined by the Lowry method (12). Error bars are SD (n = 6).

examined. Overall, protein content from mucosal scrapings was higher in the PSI and DSI compared with the cecum and colon (see Fig. 7B).

Incubation of dexamethasone-β-D-glucuronide with homogenates of the remaining muscle layers showed lower activity than in the corresponding mucosal scraping (specific hydrolytic activity, 0.07–0.14 nmol min^{-1} mg^{-1} at pH 4.5 and 0.03–0.07 nmol min^{-1} mg^{-1} at pH 6.5, respectively) and was found to be relatively constant along the entire GI tract of all investigated groups.

Permeability values measured in the Caco-2 monolayers as the transport barrier were 0.44×10^{-6} cm/sec for dexamethasone-β-D-glucoside, 1.2×10^{-6} cm/sec for dexamethasone-β-D-glucuronide, and 27×10^{-6} cm/sec for dexamethasone, respectively (Table I).

DISCUSSION

In Vitro Hydrolysis Studies

Several types of prodrugs have been designed to carry drugs to the large intestine. The delivery system examined herein is based on drug glycosides and bacterial glycosidases. The latter cleave the bond between the sugar and the aglycone to release the active agent. This concept was studied initially using glucoside prodrugs in the rat (8,9) and subsequently in the guinea pig (7). While measurable amounts of dexamethasone were delivered to the guinea pig cecum and colon using the prodrug dexamethasone-β-D-glucoside, the efficiency of delivery was less than expected (7). Several factors could be responsible for the observed deficiency of delivery: premature hydrolysis due to bacterial or host β-D-glucosidases or direct absorption of the prodrug from the lumen of the gastrointestinal tract.

In the present study, the presence of both mammalian and bacterial glycosidases was demonstrated in the rat GI tract. Overall, the greatest hydrolytic activity was observed in the luminal contents of the large intestines of conventional rats. The comparatively low hydrolytic activity in the lumen of the small intestines and tissues should allow delivery of glycoside prodrugs to the large intestines; however, in the rat, it appears that some portion of the dose would be hydrolyzed prior to reaching the large intestine. β-D-Glucuronidase activity in the lumen of the small intestine was lower than that of β-D-glucosidase activity and there was a sharper gradient of activity between the DSI and the cecum. These findings suggest that β-D-glucuronidase prodrugs would be less susceptible to premature hydrolysis in the small intestine than β-D-glucoside prodrugs.

Higher levels of β-D-glucosidase activity in the lumen of the PSI compared to the DSI is most likely due to exfoliated mucosal cells (20) as could be shown by the distribution of the hydrolytic activity in the different parts of the GI tract of germfree rats. Assuming that all the luminal β-D-glycosidase activity in the lumen of the germfree rat is mammalian in origin (e.g., brush border-originated β-D-glucosidase), we can estimate the relative contribution of bacterial and mammalian β-D-glycosidase activity in the lumen of the rat gastrointestinal tract. Based on the data obtained from the hydrolysis studies with the model substrates, the relative contributions of mammalian β-D-glucosidase activity in PSI and DSI of conventional rats were 76 and 88%, respectively. Mammalian β-D-glucuronidase activity in the PSI was about 60% of the total activity measured, while in the DSI it provided a negligible contribution. In the case of the prodrug dexamethasone-β-D-glucuronide, the PSI luminal activity in the germfree rats was 82% of that in the conventional rats, while the DSI luminal activity was 37% of that in the conventional rat. The relative levels of mucosal β-D-glucosidase and β-D-glucuronidase were very similar in the conventional and germfree rats, indicating that the mammalian glycosidase activity in the germfree rats can be used with acceptable confidence for estimating relative luminal levels in the conventional rats.

It is important to understand the effect of disease state on any drug delivery system. Therefore, the potential effect of colitis on this delivery system was examined using the acetic acid-induced colitis model (10). Colitis is induced by short mucosal exposure to a dilute solution (4%) of acetic acid. This treatment results in an acute inflammatory response in the mucosa and the submucosa within 24 hr. While reproduction of ulcers is nearly 100% reliable, the influence of the acetic acid treatment on microbial population and enzyme levels in the large intestine is unknown. It is unclear why luminal β-D-glucuronidase activity in the cecum and colon was decreased in colitic rats, while the luminal β-D-glucosidase in the large intestine was equivalent to that in conventional rats. Despite the apparent reduction in luminal β-D-glucuronidase activity, there was still a substantial gradient of activity between DSI and cecum in the colitic rats. In humans, IBD patients exhibit variable (21–23) glycosidase activity compared with healthy individuals.

The location of mammalian glycosidases is an important consideration when designing glycoside prodrugs for colonic drug delivery. β-D-Glucuronidase activity in the mucosa and muscle layers of the gastrointestinal tract appears to be lysosomal in origin based on pH activity data and the findings of others (16,17). To affect drug delivery, the glucuronide prodrugs would need to enter not only the cell, but also the lysosomal compartment, in order to be hydrolyzed *in vivo*. In addition, β-D-glucuronidase activity from lysed exfoliated mucosal cells will be reduced in the less acidic luminal contents. Incubations of mucosal GI tract scrapings of colitic rats with *p*-NP-glrd showed decreased β-D-glucuronidase activity in the inflamed regions compared to the conventional animals. Incubation of dexamethasone-β-D-glucuronide with similar homogenates (i.e., mucosal scrapings of cecum and colon of conventional and colitic rats, respectively) did not

Table I. Physicochemical Properties and Permeability (*P*) in Caco-2 Monolayers

Compound	MW	log*K*	*P* ($\times 10^{-6}$ cm/sec)[a]
Dexamethasone	392.5	1.77	27.2 ± 1.15
Dexamethasone-β-D-glucoside	554.6	0.58	0.44 ± 0.06
Dexamethasone-β-D-glucuronide (sodium salt)	590.6	−1.59	1.15 ± 0.06

[a] Mean ± SE *P* for both glycoside prodrugs is significantly different from that of dexamethasone (Fisher's Protected Least Significant Difference; $P < 0.0001$); there is no difference statistically between the *P* values of the two prodrugs.

show lower β-D-glucuronidase activities in the inflamed GI tract mucosa. Mucosal scrapings of the large intestines of these two groups exhibited equal or slightly higher activity at pH 6.5 compared to pH 4.5. Therefore, these homogenates may have been contaminated with bacterial β-D-glucuronidase and changes in the lysosomal β-D-glucuronidase activity due to the inflammatory process were not measurable.

The protein content in the various GI segments examined was relatively constant, with several exceptions (see Fig. 7), from region to region. This finding is important due to the use of protein content of the homogenates to normalize the specific enzyme activity data collected. Data normalized against protein content would be misleading if there was marked variance of protein content from region to region.

It has been suggested that the location of mammalian β-D-glucosidase is the brush border (24,25); a more recent study suggests that β-D-glucosidase activity is located primarily in the cytosol of enterocytes rather than the brush border (26). It was observed in these studies that homogenates of rat GI tract luminal contents possess substantial β-D-glucosidase activity. Assuming that cell lysis occurs or that these enzymes are located on the surface of sloughed enterocytes, these enzymes will probably prematurely hydrolyze β-D-glucoside prodrugs.

The large intestine, when populated with gut microflora, exhibits a negative oxidation reduction potential of about −200 mV (27,28). However, the present hydrolysis studies were not performed under anaerobic conditions because it was found that the presence or absence of oxygen did not alter the rate of hydrolysis of the prodrug dexamethasone-β-D-glucoside in homogenates of cecal contents of the guinea pig (19). In general, measurement of glycosidase activity of gut microflora is performed without the exclusion of oxygen.

The rat, as well as many other rodents and laboratory animals, has substantially higher glycosidase activity in the small intestine compared with that of humans (29). Thus, the human GI tract has a much sharper gradient of bacterial colonization and hence glycosidase activity. The prodrug approach should therefore be more efficient in humans than can be demonstrated in the laboratory rat.

Permeability in Caco-2 Monolayers

The objective of the permeability study was to evaluate the absorption potential of dexamethasone and its hydrophilic prodrugs. Permeability values in Caco-2 monolayers demonstrated for compounds that are generally completely absorbed from the human GI tract are $>70 \times 10^{-6}$ cm/sec. Compounds with poor absorption (<20%) have permeability values $<10 \times 10^{-6}$ cm/sec. Thus, the absorption potential for the investigated prodrugs is negligible. Moreover, the permeability values for the prodrugs and the nonabsorbable marker PEG 4000 are similar (30). Therefore, we conclude that converting dexamethasone to its prodrugs prevents its absorption until cleavage. In addition, there was no evidence of hydrolysis of either dexamethasone product during its permeation through the Caco-2 monolayers.

CONCLUSIONS

The prodrug dexamethasone-β-D-glucuronide appears to be a suitable candidate to deliver dexamethasone (or other corticosteroids possessing a hydroxyl group at the C-21 position) to the large intestine. Compared with glucosides, the glucuronide prodrugs should be more stable in the small intestine. The prodrug, according to permeability measurements in Caco-2 monolayers, should be poorly absorbed from the GI tract. The relatively sharp gradient in enzyme activity between the large and the small intestine should be sufficient to hydrolyze the prodrug to the drug, which can then be absorbed from the lumen of the colon.

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