Menthol-β-D-Glucuronide: A Potential Prodrug for Treatment of the Irritable Bowel Syndrome

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Received March 28, 1994; accepted June 17, 1994

Menthol-β-D-glucuronide is a potential prodrug for colonic delivery of the spasmolytic agent menthol. Menthol is the primary constituent of peppermint oil, which is used to treat the irritable bowel syndrome. The chemical stability of menthol-β-D-glucuronide was assessed at various pHs (1.5, 4.5, 6.0 and 7.4) over a 4 to 24 h period at 37°C. The prodrug was stable, i.e., there was less than 0.1% hydrolysis of the prodrug, at pHs of 4.5, 6.0 and 7.4. At pH 1.5, the prodrug was about 20% hydrolyzed over a 4 h period suggesting the need for an enteric coating to prevent premature hydrolysis in the stomach. The stability of the prodrug was also assessed in luminal contents of the laboratory rat and in human stool samples. These studies were performed at concentrations designed to assess relative velocities of hydrolysis (i.e., substrate concentrations in excess of the K_m). The prodrug was stable in luminal contents of the rat stomach, proximal small intestine, and the distal small intestine. The rate of hydrolysis of menthol-β-D-glucuronide was 6.26±2.88 nmol min^{-1} mg^{-1} and 2.34±1.22 nmol min^{-1} mg^{-1} in luminal contents of the rat cecum and colon, respectively. The hydrolysis rate of menthol-β-D-glucuronide was lower in human stool samples (0.52±0.46 nmol min^{-1} mg^{-1}). The prodrug had a measured log octanol/buffer partition coefficient of −1.61 suggesting it should be poorly absorbed from the lumen of the gastrointestinal tract. The data support the hypothesis that menthol-β-D-glucuronide is a candidate for the delivery of menthol to the large intestine under *in vivo* conditions.

KEY WORDS: irritable bowel syndrome; spastic colon; menthol; glucuronide prodrug; colonic drug delivery.

INTRODUCTION

The irritable bowel syndrome (IBS), including spastic colon, is a wide-spread problem. It is considered to be a functional disorder of abdominal pain with defecation or a change in bowel habit (1). It is estimated that 8 to 24% of healthy individuals in the United States and the United Kingdom experience spastic colon (2). Pharmacologic treatment of IBS is complicated due to a variety of factors including a strong placebo effect (1). Nonetheless, pharmacotherapies that control various symptoms of IBS have been developed (3).

The use of carminative agents, such as peppermint and related essential oils, to control muscle contractions has been studied for many years (4,5). Peppermint oil has been used to reduce lower esophageal sphincter pressure (6). More recently, the ability of peppermint oil to control spasms and control colonic motility in the large intestine has been reported. Duthie concluded that peppermint oil may be useful in the treatment of IBS based on its effects on colonic motility in humans (7). Rees et al (8) reported that peppermint oil was useful in the treatment of IBS when administered in an enterically coated dosage form. In the absence of the delayed release action of the enteric coating, orally administered peppermint oil produces relaxation of the cardioesophageal sphincter rather than smooth muscles in the colon. In addition, local administration of peppermint oil normally leads to rapid and transient effects (9). Two enterically coated peppermint oil products are available in the UK (Colpermin® and Mintec®); these products have been evaluated pharmacokinetically in human subjects (9,10). In addition to delivering more active agent to the large intestine, these dosage forms may also control the release of peppermint oil over an extended period of time.

The primary component of peppermint oil is menthol. This carminative agent composes at least 45% of the total material present in commercially available preparations (11). Menthol has been used for many years in a wide array of pharmaceutical and consumer products. For instance, menthol produces a local anesthetic effect and is an anti-irritant in topical compositions such as those used to treat acne vulgaris, seborrhea, and athletes foot. Menthol is also used as an inhalant for treating pulmonary congestion associated with colds, allergies, and other upper respiratory conditions.

When peppermint oil is orally administered, menthol is rapidly absorbed and is then metabolized to its glucuronide salt. Menthol-glucuronide is excreted nearly completely in the urine, where it is commonly used as a measure of the bioavailability of menthol and peppermint oil (10,11). Oral administration of this metabolite (menthol-β-D-glucuronide) should lead to delivery of menthol to the large intestine where it would then be hydrolyzed by bacterial β-D-glucuronidases. Hence delivery of menthol to the colon should be possible in the manner demonstrated with steroid-glucuronides useful in the treatment of ulcerative colitis and Crohn's colitis (12–14).

This study presents data collected under *in vitro* conditions used to evaluate the potential of prodrug menthol-β-D-glucuronide to deliver menthol to the colon and the rate of release once the prodrug reaches the colon. These studies were conducted using luminal contents of the laboratory rat as well as human stool samples.

MATERIALS AND METHODS

Materials

(−) Menthol and menthol-β-D-glucuronide (ammonium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclohexanol was obtained from Aldrich Chemical Co. (Milwaukee, WI). All reagents were used as received and were of the highest purity available. Human fecal samples were obtained through Dr. Richard Fedorak of the Division of Gastroenterology, University of Alberta. The samples were collected from normal volunteers; no attempt was made to control diet. The samples were collected in plastic

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0724-8741/94/1200-1707$07.00/0

bags and sealed immediately. The samples were quickly frozen at −70°C until use.

Animals

Male Sprague Dawley rats (200–279 g, 6 to 7 weeks old) were obtained from Simonsen Labs (Gilroy, CA) and were housed in the animal care facility at SRI International. The 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 gastrointestinal tract was isolated. Following removal of fatty and mesenteric tissues, the gastrointestinal tract was segmented into stomach, small intestine, cecum, and colon. The small intestine was further divided into proximal and distal segments of equal length (PSI and DSI, respectively). Then the whole gastrointestinal tract segments were immediately quick-frozen and stored at −30°C until use. Previous experiments had shown that freezing of gastrointestinal 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.

Hydrolysis of Menthol-β-D-Glucuronide in Solution

The hydrolysis of the prodrug menthol-β-D-glucuronide was assessed at 37°C at pH 1.5, 4.5, 6.5, and 7.4. All solutions were prepared using phosphoric acid, sodium phosphate, or disodium phosphate at 0.1 M. The prodrug (menthol-β-D-glucuronide, ammonium salt) was prepared as a standard solution of 130 mM and 21 μL of the appropriate buffer solution was added to 79 μL of the prodrug solution in 13 × 100 mm test tubes to make solutions of 100 mM of prodrug. The tubes were capped immediately after addition of menthol-β-D-glucuronide to the buffer solution. The samples, run in duplicate, were shaken and incubated for 0, 1, 2, 4, and 24 h. At the appropriate times, the samples were removed from the water bath and 100 μL of the internal standard cyclohexanol (100 mM in acetone) and 1.0 mL of saturated sodium chloride solution were added to each tube. The samples were thoroughly mixed and then 4.0 mL ethyl ether was added. The samples were vortexed for 30 s and then centrifuged (500*g*, 3 min) at room temperature. A portion of the supernatant (5 μL) was injected onto the gas chromatography column (see below for conditions used).

In Vitro Hydrolysis Studies in Intestinal Contents

Preparation of Rat Luminal Content Homogenates. Luminal contents from the laboratory rat (Sprague Dawley strain) were used. Following sacrifice and collection of luminal contents (see above), freshly isolated or thawed gastrointestinal 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 20% slurries, and cecal and colonic contents to 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 minutes at 4°C) to remove particulate material.

Preparation of Human Fecal Samples. One gram of feces was removed from the frozen fecal samples, placed in a 6 dram vial, and allowed to thaw. To each sample was added 4.0 mL of 0.1 M $NaHPO_4$, pH 6.5. The samples were vortexed for 30 s to mix the feces with the buffer. Previous experiments showed hydrolysis of glucuronide prodrugs was equal either when whole fecal samples (unhomogenized) or homogenized/centrifuged fecal samples were used. Therefore, these studies were conducted in unhomogenized feces.

Incubation Conditions. The enzyme kinetics for hydrolysis of the prodrug menthol-β-D-glucuronide were measured in rat cecal contents. The substrate (menthol-β-D-glucuronide), dissolved in 0.9 mL, was incubated with 0.1 mL rat cecal homogenate (10% homogenized slurry) for 30 min at concentrations ranging from 0 to 100 mM. After 30 min, saturated NaCl (1.0 mL) was added to the reaction and then 100 μL of a 112.4 mM cyclohexanol was added. After thoroughly mixing, ethyl ether (4 mL) was added and the solution mixed for 30 s by vortexing. The samples were centrifuged three time (500*g*, 3 min) and 5 μL injected onto the GC column for analysis (see below for details).

Based on results of the experiment described above, the hydrolysis of menthol-β-D-glucuronide was studied under enzyme-saturated conditions. The substrate (menthol-β-D-glucuronide), dissolved in 0.9 mL buffer (0.1 M sodium phosphate, pH 6.5), was incubated with 0.1 mL rat gastrointestinal content homogenates (10% homogenized slurry) for 30 min at 100 mM. After 30 min at 37°C, saturated NaCl (1.0 mL) was added to stop the reaction and then 100 μL of a 99.5 mM cyclohexanol was added. After thoroughly mixing, ethyl ether (4 mL) was added and the solution mixed for 30 s by vortexing. The samples were centrifuged three time (500*g*, 3 min) and 5 μL injected onto the GC column for analysis (see below for details).

Studies involving human fecal samples were performed in a manner similar to those described above for the rat gastrointestinal contents. A 127 mM solution (0.79 μL) was added to the fecal suspension (20 wt%; 0.21 μL) in 13 × 100 mm test tubes to create a 100 mM substrate concentration. The suspension was then mixed thoroughly. After 30 min at 37°C, saturated NaCl (1.0 mL) was added to stop the reaction and then 100 μL of a 99.5 mM cyclohexanol was added. After thoroughly mixing, ethyl ether (4 mL) was added and the solution mixed for 30 s by vortexing. The samples were centrifuged three time (500 *g*, 3 min) and 5 μL injected onto the GC column for analysis.

Determination of n-Octanol/Buffer Partition Coefficients

The log octanol/buffer partition coefficient of menthol-β-D-glucuronide was measured by first preparing a stock solution in buffer (0.05 M phosphate, pH 7.4 saturated with 1-octanol) and measuring the absorbance at 192 nm. Initial concentration of the prodrug in the buffer was 0.83 mg/mL. Next, three different concentrations of the prodrug in buffer (0.83, 0.63, and 0.42 mg/mL) (2 mL) were mixed with 1-octanol (50 mL) at room temperature by constantly inverting a 125 mL Erlenmeyer flask for 5 min. The concentration of menthol-β-D-glucuronide remaining in the buffer phase was measured at 192 nm. The log octanol/buffer partition coefficient (log *K*) was then calculated by dividing the concentra-

tion of compound in the octanol phase (determined by difference) by the concentration in buffer. The log K of menthol was calculated from oil/water data according to Leo et al. (15).

Analytical Methods

Menthol was measured in all hydrolysis experiments using a gas chromatographic technique. The equipment used was a Hewlett-Packard Model 5890, Series II GC equipped with a FID detector. The column used was fused silica capillary (J & W, Folsom, CA) 30 m × 0.241 mm. Injector and detector temperatures were 250°C. The column oven was set at 60°C for one min and raised at 15°C/min to 150°C and held there for 3 min over the course of the run. A total of 5 μL of sample was injected; menthol had a retention time of 5.9 min while that of the internal standard, cyclohexanol, was 2.8 min. Standards over the range of 0.25 to 2.5 mM were found to be linear.

RESULTS AND DISCUSSION

Hydrolysis of Menthol-β-D-Glucuronide

The hydrolysis of the prodrug menthol-β-D-glucuronide in solution was evaluated at several pHs (1.5, 4.5, 6.5, and 7.4) up to 24 h at 37°C. This range represents possible pHs that could be encountered during transit throughout the human gastrointestinal tract. The prodrug was stable at pHs 4.5, 6.5, and 7.4 tested up to 4 to 24 h: less than 0.1% of the prodrug was hydrolyzed to menthol at any pH over these times. At pH 1.5, the prodrug was about 20% hydrolyzed over a 4 h period at 37°C. Based on this finding, the dosage form will probably be required to prevent exposure of the prodrug in the acid environment of the stomach through the use of an enteric coating or other formulation approach.

The hydrolysis of the prodrug menthol-β-D-glucuronide was evaluated in rat cecal homogenates at a variety of substrate concentrations to determine the K_m of the hydrolysis reaction. This site in the rat gastrointestinal tract has the highest level of β-D-glucuronidase activity as determined with the model substrate *p*-nitrophenyl-β-D-glucuronide (12) relative the other regions (stomach, PSI, DSI, colon). The K_m for hydrolysis of menthol-β-D-glucuronide in homogenates of rat luminal contents was found to be 10 mM. Therefore, the concentration of the substrate in all subsequent hydrolysis experiments was 100 mM to ensure that all reactions were measured at V_{max}.

The hydrolysis of menthol-β-D-glucuronide in homogenates from various regions of rat gastrointestinal tract contents was evaluated to determine relative rates of hydrolysis. A key aspect of colonic delivery of drugs relying on the prodrug approach is the relative stability of the prodrug in the stomach and proximal small intestine (PSI), distal small intestine (DSI), and the relative instability in the cecum and colon. The rates of hydrolysis (nmol min^{-1} mg^{-1} of intestinal homogenate) were measured in the stomach, PSI, DSI, cecum, and colon. Figure 1 shows the data collected. There was no measurable amount of prodrug hydrolyzed in the luminal contents of the rat stomach, PSI, or DSI. This finding is in contrast to previous studies involving glycoside prodrugs wherein some hydrolysis was demonstrable in the luminal contents from the upper gastrointestinal tract of rats and guinea pigs (12–14). As expected, the prodrug was susceptible to hydrolysis in luminal contents of the cecum and colon. In most mammals, the level of bacterial β-glucuronidase activity is considerably higher in the large intestine compared with the stomach and small intestine. This distribution of enzyme activity is due to the relatively large number of bacteria resident in the distal intestine (16).

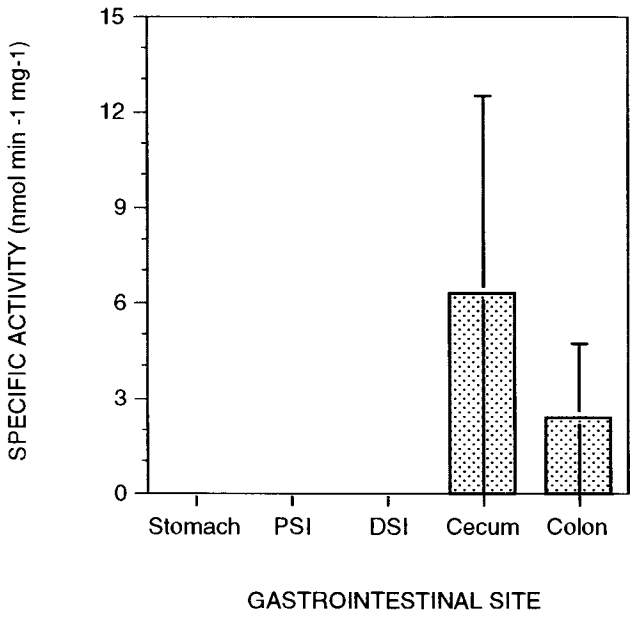

Fig. 1. Hydrolysis of the prodrug menthol-β-D-glucuronide (nmoles of substrate hydrolyzed per min per mg of luminal contents) measured from various locations in the rat gastrointestinal tract. There was no menthol detected over the the one hour experiment in the luminal contents of the stomach, PSI, or DSI. Error bars are mean±S.D. (n = 6).

The hydrolysis of the prodrug menthol-β-D-glucuronide in human stool samples was measured under conditions similar to those used with the rat. However, in the case of the human stool samples, they were not homogenized prior to measuring hydrolysis. It has been found that the majority (80–90%) of glycosidase activity is extracellular or associated with clumps of solid matter present in the gastrointestinal tract (16). The rate of hydrolysis of the prodrug menthol-β-D-glucuronide was measured in human stool samples at 0.52 ± 0.46 nmol min^{-1} mg^{-1} (mean ± S.D, n = 4). This value is somewhat less than that measured in luminal content homogenates from the rat colon [2.34 ± 1.22 nmol min^{-1} mg^{-1} (mean ± S.D., n = 6)].

Partition Coefficient of Menthol-β-D-Glucuronide

The log octanol/buffer partition coefficients (log K) of the prodrug menthol-β-D-glucuronide and menthol are shown in Table 1 along with those of several other glucuronide and glucoside prodrugs. There was a substantial (over 4 order of magnitude) decrease in log K when ammonium glucuronate is conjugated to menthol. Such a large reduction in log K should substantially limit absorption from the gastrointestinal tract.

Table I. Physicochemical Properties (Molecular Weight and Log Octanol/Buffer Partition Coefficient) of Menthol, Its Glucuronide Prodrug, and Several Related Prodrugs and Respective Aglycones[a]

Compound	Molecular weight	Log K
Menthol[b]	156.3	3.07
Menthol-β-D-glucuronide (ammonium salt)	349.4	−1.61
Dexamethasone	392.5	1.77
Dexamethasone-β-D-glucoside	554.6	0.58
Dexamethasone-β-D-glucuronide (sodium salt)	590.6	−1.59

[a] Data on dexamethasone and its prodrugs obtained from ref. 10.
[b] Calculated from oil/water system according to ref. 13.

Colonic Delivery of Menthol for Treatment of IBS

The data presented in this paper generally support the concept that menthol can be delivered to the mammalian large intestine using the glucuronide prodrug of menthol. Menthol-β-D-glucuronide should be poorly absorbed from the lumen of gastrointestinal tract due to its larger size and greatly reduced octanol/buffer partition coefficient. At least in the rat, the prodrug appears to be stable in the stomach and small intestine. In the cecum and colon, however, the prodrug is hydrolyzed at rate ranging from 3–6 nmol min^{-1} mg^{-1} of intestinal contents. Hydrolysis rates in the human large intestine appear to be about 5 fold less than that found in the rat colon assuming the hydrolysis data from the stool samples are representative of the large intestine *in vivo*.

In the previous studies using glucoside and glucuronide prodrugs of corticosteroids (12,14), both mammalian and bacterial glycosidases were demonstrated in the rat GI tract. While not evaluated in the present study, there appears to be very little if any activity from either source in the rat small intestine when the substrate is menthol-β-D-glucuronide. Consistent with previous studies was the relatively high hydrolytic activity observed in the luminal contents of the large intestines of rats. Low or nonexistent hydrolytic activity in the lumen of the small intestines and tissues should allow efficient delivery of glycoside prodrugs to the large intestines; this prerequisite appears to be met in the case of menthol-β-D-glucuronide.

Patients with IBS often experience painless diarrhea and other transit abnormalities (2). If decreased transit times occur in the large intestine, it could alter the pharmacokinetics of a drug delivery system relying on bacterial glucuronidase activity in the large intestine. It is at present difficult to estimate the impact of severe diarrhea on the performance of the proposed delivery system.

The large intestine, when populated with gut microflora, exhibits a negative oxidation reduction potential of about −200 mV (17,18). However, the present hydrolysis studies were performed under aerobic conditions because it was found that the hydrolysis of the prodrug dexamethasone-β-D-glucoside was unaltered by presence or absence of oxygen in homogenates of cecal contents of the guinea pig (14). 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 reportedly substantially higher glycosidase activity in the small intestine compared with that of humans (19). Thus, the human gastrointestinal tract has a much sharper gradient of bacterial colonization and hence glycosidase activity. As noted above, there were undetectable levels of β-glucuronidase activity in the lumen of the rat small intestine. Thus, the rat, fed a normal raw chow diet, would appear to be a satisfactory model for delivery of menthol from its glucuronide prodrug.

In conclusion, the glucuronide prodrug of menthol is stable in the stomach and small intestine of the rat and is hydrolyzed at a rate of about 3–6 nmol min^{-1} mg^{-1} of large intestinal contents. The rate of hydrolysis in human stool samples is hydrolyzed about 5–10 times more slowly in human stool samples compared with that in the rat cecum or colon. The prodrug has a small log K (−1.61) and should therefore be poorly absorbed from the lumen of the GI tract. Thus, menthol-β-D-glucuronide has the potential to deliver the carminative agent menthol to the large bowel in an efficient and effective manner.

ACKNOWLEDGMENTS

We would like to thank Doris Taylor for measurement of partition coefficients.

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