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The Impact of Handling and Storage of Human Fecal Material on Bacterial Activity

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ABSTRACT

Fecal material prepared from human stools is frequently used for the assessment of bacterial degradation of active pharmaceutical ingredients as relevant data are useful for evaluating the potential for colonic drug delivery. The impact of handling and storage of human fecal material on bacterial activity was assessed by evaluating the degradation characteristics of metronidazole and olsalazine. Multiple freeze (−70°C)–thaw cycles should be avoided. Incubation of frozen material for about 2 h in the anaerobic workstation ensures regeneration of the highest possible bacterial activity. Material could be stored at −70°C for at least 12 months.

Introduction

Various *in vitro* setups have been proposed for the assessment of bacterial degradation of active pharmaceutical ingredients (APIs),[1] and relevant data are very useful for evaluating the potential for colonic drug delivery.[2] However, literature data on the impact of handling and storage of human fecal material on bacterial activity are inconclusive, whereas the effect of handling and storage of human fecal material on API degradation kinetics are very limited.

The impact of freezing (−80°C) on bacterial activity in human fecal material was found to be nonsignificant, based on the short-chain fatty acid production and β-glucuronidase and β-glucosidase activities.[3,4] In contrast, after freezing, *Escherichia coli*, *Enterococcus*, and *Bacillus* behaved differently to UV irradiation compared to those not frozen.[5] Furthermore, repeated freeze-thaw cycles had nonsignificant effect on the response of freeze-treated *Enterococcus faecallis* bacteria to UV,[6] but it led to significant reduction of fecal streptococci, and antibiotic-resistant bacteria.[7] Cooling and thawing rates as well as duration of storage of frozen bacteria are additional issues for consideration. Various ranges of optimum cooling and thawing rates for *E. coli* have been suggested.[8] On the other hand, based on data with ximelagatran (an anticoagulant), a trend for decreased bacterial activity has been reported, after 6 months of storage of fecal material at −70°C.[9] Similarly, total number of antibiotic-resistant bacteria decreased slowly but constantly over the 10-month freezing period at −18°C.[7] In contrast, however, storage of eight enteropathogenic species at −70°C (*E. coli*, *Salmonella typhimurium*, *Shigella flexneri*, *Shigella sonnei*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Vibrio cholerae*, and *Aeromonas hydrophyla*) effectively preserved all organisms for 12 months, except *C. jejuni*.[10]

In this study, we evaluated the impact of handling and storage of human fecal material on bacterial activity by focusing primarily to 2 enzymes which are produced by most bacteria in the healthy human gut flora, nitroreductase, and azoreductase (Supporting Information). Enzyme activity was assessed indirectly by evaluating the degradation characteristics of 2 substrates of nitroreductase and azoreductase, metronidazole[11] and olsalazine,[12] respectively, which are chemically stable in the range of pH values that were achieved in human fecal material prepared and tested in this study ($5.5 < pH < 6.5$).[13]

There were 3 specific objectives for this study. The first was to optimize the incubation period of frozen fecal material in the anaerobic workstation before evaluating bacterial activity. The

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second objective was to evaluate the impact of second freeze-thaw cycle on bacterial activity. The third objective was to evaluate the impact of storage period at −70°C on bacterial activity.

Materials and Methods

Materials

Metronidazole was purchased from Sigma-Aldrich (St. Louis, MO) and olsalazine from Farchemia (Treviglio BG, Italia). Acetonitrile and water were of HPLC grade from Sigma-Aldrich. All other chemicals were of analytical grade. Sterile normal saline for IV infusion was used (Demo Pharmaceuticals, Krioneri, Greece).

Human Subjects

Stools were collected from 6 healthy male adults (28-57 years old), after receiving approvals by the Bioethics Committee of the Department of Pharmacy, National and Kapodistrian University of Athens (AP 524; 22 December, 2014). Because composition of flora is influenced by age, diet, and socioeconomic conditions,[14] the following criteria were applied for enrolling a subject.

Inclusion Criteria

Willingness of the subject to participate as indicated by his signed informed consent, aged 18-60 years, and weight within 20% of ideal body weight as determined by Metropolitan Life Tables. Willingness to abstain from smoking and drinking alcohol for at least 3 days before the day of stool collection. Nutrition habits not deviating much from the Mediterranean diet (olive oil, fish, meat, dairy products, legumes, fruits, vegetables). No treatment with antibiotics during the last 6 months before stool collection.[15] No probiotics or prebiotics (food supplements, functional foods, etc.) for the last 3 months.[16] No use of laxatives or cathartics for the last 7 days.[17] Regular intestinal habits and stools consistency of type 2, 3, or 4, according to Bristol stool scale[18] for at least a month before the day of stools collection.

Exclusion Criteria

Existence of a major health problem (cardiovascular, pancreatic, hepatic, thyroid, etc.) and/or existence of any condition requiring prescription drug therapy, recent history of gastrointestinal symptom regardless of the severity (e.g., heartburn, constipation, hemorrhoids, etc.), use of medication during the last 2 weeks before stool collection, exclusion of a specific food category from his diet (e.g., meat or dairy products), or irregular bowel habits.

Preparation of Fecal Material

On the day of stool collection, the subject was allowed to eat breakfast and lunch as usually, and stools were collected during the first defecation of the day. Stools were collected in preweighed plastic containers and were transferred into the anaerobic workstation (37°C; relative humidity 70%; atmosphere of 80% N_2, 10% H_2, and 10% CO_2[9,13,15]) in less than 10 min after defecation. In the anaerobic workstation, stools were weighed and diluted 1:3.8 w/v with normal saline, to prepare fecal material with a composition of approximately 25% w/v.[9,13,19] Fecal material was then homogenized using a mixer, and the slurry was sieved through a metal mesh with a pore size of 350 μm (Retsch GmbH, Haan, Germany) to remove any heterogeneous fibrous material.[9,15,20] Depending on the amount of stools of each subject, 60-80 screw-cap glass vials (capacity 20 mL) were filled with homogenated fecal material. Vials were sealed, transferred out of the anaerobic workstation, and stored at −70°C. Stools weight varied from 106 to 337 grams among volunteers. Stool content of individual fecal materials ranged from 23.6% to 28.6%, with a mean (SD) of 25.5 (1.7)%. Fecal material from each vial was used only once.

Optimization of Incubation Period of Frozen Fecal Material

To optimize the duration of incubation in the anaerobic workstation before evaluating bacterial activity, frozen fecal material from one subject ($n = 3$ vials) was incubated for 0.5 and 2 h before initiating the experiment with metronidazole. In addition, pooled fecal material (prepared by using equal volumes of the 6 individual fecal materials) was incubated for 2 and 36 h before initiation of the experiment with metronidazole and for 2 and 24 h before the initiation of the experiment with olsalazine. One to 3 vials with pooled fecal material were used in these experiments.

Impact of Second Freeze-Thaw Cycle of Fecal Material on Bacterial Activity

Degradation of metronidazole was studied, after the first and after the second freeze-thaw cycle by using fecal material from 1 volunteer ($n = 1$-3 vials). Degradation of olsalazine was studied in all 6 individual fecal materials. On exiting the freezer for the first time, each vial was incubated in the anaerobic workstation for 2 h before initiating a degradation experiment. Then, the vial was returned to −70°C and stored for an extra week. On exiting the freezer for the second time, the vial was brought again in the anaerobic workstation and, after 2 h, the degradation experiment was initiated.

Impact of Storage Period of Frozen Fecal Material on Bacterial Activity

Degradation of metronidazole and olsalazine in the 6 individual fecal materials was studied within 1 week and after 1, 3, 6, 8, 10, and 12 months of preparation and storage at −70°C. On exiting the freezer for the first time, each vial was incubated in the anaerobic workstation for 2 h before initiating a degradation experiment. At each time, individual fecal materials were tested in triplicate ($n = 3$ vials).

Evaluation of Bacterial Activity

All experiments were performed in the anaerobic workstation under the same conditions used for preparing the fecal materials. Stock solutions of metronidazole and olsalazine were prepared in normal saline. The initial concentration of the stock solution of metronidazole was 100 μg/mL, whereas of olsalazine, it was 140 μg/mL.[13] One hundred twenty-five microliters of the API solution was added in 1 mL of fecal material and the mixture was incubated in a thermomixer at 37°C with oscillations of 1000 rpm (Eppendorf Thermomixer comfort; Eppendorf AG, Hamburg, Germany). Sampling time points for metronidazole were 0, 5, 10, 15, 20, 30 min, whereas for olsalazine, they were 0, 10, 20, 30, 60, 90, 120 min.[13] One hundred microliters of sample were withdrawn from the mixture at each sampling point, and 300-μL ice cold acetonitrile was added to terminate bacterial activity. The sample was then transferred out of the anaerobic workstation for assaying metronidazole or olsalazine content. Samples were centrifuged [10°C, 10 min, 10,000 rpm (Hettich Micro 200; Hettich, Tuttlingen, Germany)], and 100 μL of the clear supernatant was diluted with 900 μL of mobile phase. After vortexing, quantification was achieved with HPLC by using standard curves constructed in fecal material (0.01-0.5 μg/mL).