Degradation of Bromazepam by the Intestinal Microflora

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Degradation of four benzodiazepines, i.e. bromazepam, diazepam, desmethyldiazepam, and 7-bromo-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2(1H)-one, in a broth containing human feces was studied. Degradation of bromazepam was also investigated in a fecal medium, a homogenate of the liver and a homogenate of intestinal epithelial cells of rat. More than 80% of bromazepam, which contains a pyridine ring in its structure, was degraded in the broth containing 8% human feces under both anaerobic and aerobic conditions. The major degradation product of bromazepam was identified as 2-(2-amino-5-bromobenzoyl)pyridine by means of thin layer chromatography and gas chromatography-mass spectra analyses. About 70, 90, and 90% of bromazepam remained in the fecal medium, homogenate of the liver and homogenate of intestinal epithelial cells of rat, respectively, after incubation for 5 h at 37 °C.

Therefore, degradation of bromazepam by the intestinal microflora may be considered to be one of the major factors influencing the bioavailability after oral administration to man.

Keywords—bromazepam; degradation; fecal flora; gut flora; intestinal microflora; rat

Recently, degradation of imipramine,1) digoxin,2,3) and nitroglycerin4) by the intestinal microflora has been investigated in order to explain their low extents of bioavailability after oral administration to man. In the case of sulfinpyrazone,5) the distal gut with its microflora is the principal and possibly the only site of reduction of sulfinpyrazone to its active form in humans, and metabolism of the drug by the gut flora is essential to its pharmacological response. Bromazepam, one of the 1,4-benzodiazepine antianxiety agents, has also been reported to exhibit low bioavailability after oral administration. Kasama et al.6) reported that the area under the plasma concentration–time curve (AUC) value after rectal administration of 3 mg of bromazepam was equivalent to that after oral administration of 5 mg of bromazepam. The present study was undertaken to investigate the possible degradation of bromazepam in fresh human feces.

Experimental

Materials—Bromazepam was a gift from Eisai Co., Tokyo, and diazepam, desmethyldiazepam, and 7-bromo-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2(1H)-one (BDPB) were gifts from Nippon Roche Co., Tokyo. An incubation medium (shown in Table I) was purchased from Nissui Seiyaku, Tokyo. Chemicals used were of reagent grade, and were purchased from Wako Pure Chemical Industries, Osaka.

Degradation by the Intestinal Microflora—About 100 ml of the medium containing 8% (w/v) fresh feces, obtained from normal subjects who had not taken any antibiotic prior to the experiment, was centrifuged at 2000 rpm for 5 min. An 800-μl portion of bromazepam solution in dimethylformamide (DMF) (8 mg/ml) was added to 80 ml of the supernatant of the fecal medium and the mixture was incubated at 37 °C under both anaerobic (Gaspak®, BBL Microbiology Systems, Cackeysville, Md, U.S.A.) and aerobic conditions. A 2-ml portion of the fecal medium was collected at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 h and stored at −70 °C until analysis. Degradation of drugs in the sterilized 8% fecal medium was examined as a control. Degradation of drugs in a rat fecal medium was also studied similarly.

Determination of Undegraded Drugs—A sample (2 ml) was mixed with 3 ml of 0.2 M borate buffer, pH 8.0, and 5 ml of toluene containing prazepam as an internal standard. After being shaken for 10 min, the mixture was
centrifuged at 3000 rpm for 20 min and the organic phase was then evaporated in vacuo at 60 °C. The residue was dissolved in 50 μl of methanol and a 10-μl aliquot of the resultant solution was injected into the high performance liquid chromatography (HPLC) system (LC-4A, Shimadzu Manufacturing Co., Kyoto) equipped with a ultraviolet (UV) detector set at 233 nm. A 150 mm column of Shim-pack CLC-ODS (particle size 5 μm, Shimadzu Manufacturing Co.) was employed with a mobile phase of methanol : water : ammonium hydroxide = 270 : 230 : 3 at the flow rate of 1.0 ml/min at a column temperature of 50 °C.

Decrease in bromazepam concentration with time was expressed as the ratio of peak area of bromazepam/peak area of the internal standard at the sampling time divided by that at time zero.

Identification of the Degradation Product—An extract from fecal culture medium after incubation was spotted on a silica gel plate (Kieselgel 60 F254, Kanto Chemical Co., Tokyo), developed in acetone : chloroform = 10 : 90 and visualized under UV light. The extract was also examined by gas chromatography-mass spectrum (GC-MS) analysis (QP-1000 GC-MS system, Shimadzu Manufacturing Co.). The GC part of the GC-MS system was equipped with a 1-m glass column packed with 3% OV-17 on Chromosorb Q. The flow rate of helium was 50 ml/min. The temperatures of the injection port, column, transfer line to the MS system, and ion source of the MS system were 275, 250, 280, and 280 °C, respectively. MS were obtained in an electron impact (EI) mode at an electron energy of 70 eV.

Metabolism in Homogenates of Liver and Intestinal Epithelial Cells in Rats—An 8-ml portion of 0.1 M Tris HCl buffer solution, pH 7.4 was added to 2 g each of the excised liver mass and intestinal epithelial cells of rat. Saline was introduced into the artery of the excised liver of a rat to remove blood, and the tissue was homogenized (Physcotron, Nichi-on Co., Chiba). Everted rat intestine was scraped with a slide glass to collect epithelial cells. Both mixtures were homogenized and centrifuged at 3000 rpm for 10 min. A 50-μl portion of bromazepam solution (2 mg/ml) in ethanol was added to 0.95 ml of the supernatant, and then the mixture was incubated at 37 °C. A 50-μl aliquot was collected after incubation for 0, 1, 2, 3, 4, and 5 h, and 100 μl of acetonitrile containing clonazepam as an internal standard (50 μg/ml) was immediately added to deproteinize the sample. Each mixture was centrifuged at 7000 rpm for 2 min. A 2-μl portion of each supernatant was injected into the HPLC system.

The HPLC conditions were identical to those given earlier in the section on determination of undegraded drugs. Total amounts of protein in the supernatant of homogenates of the rat liver and intestinal epithelial cells in this study were determined by the Lowry method.

Results and Discussion

Figure 1 shows the degradation profiles of four benzodiazepines in the broth containing...
8% human feces. Bromazepam was rapidly degraded in the broth at 37 °C, while the other three benzodiazepines were not degraded up to 5 h. About 80% of bromazepam was degraded within 5 h under both anaerobic and aerobic conditions, while it was not degraded in the sterilized fecal culture, indicating that degradation of bromazepam was not caused by the fecal contents themselves but by the gut microflora. The degradability of bromazepam may be related to the fact that this compound is the only one containing a pyridine ring, among those tested.

Disappearance profiles of bromazepam in homogenates of the liver, intestinal epithelial cells, and the fecal flora of rats are shown in Fig. 2. Total amounts of protein in the supernatant of homogenates of the liver and intestinal epithelial cells of rats were 29.4 and 22.5 mg/ml, respectively. Percentages of degradation of bromazepam by the liver and intestinal epithelial cells in rats were about 10% each, while that by rat feces was about 30% in 5 h. Degradation of bromazepam in homogenates of fecal cultures in rats was observed from 3 h after the initiation of incubation, presumably reflecting the time required for the growth of bacteria in a rat microfloral culture.

Kasama et al. observed lower bioavailability of bromazepam after oral administration than after rectal administration. Fukushima et al. reported that the plasma concentrations of bromazepam after rectal administration were higher than those after oral administration to 12 healthy volunteers. The AUC after rectal administration of bromazepam was about twice that after oral administration. Kasama et al. also reported on the pharmacokinetics after oral administration of 5 mg of bromazepam and rectal administration of 3 mg of bromazepam to 7 healthy volunteers. The AUC0–∞ values after rectal administration of 3 mg were equivalent to those after oral administration of 5 mg, suggesting 60% relative bioavailability of bromazepam after oral administration of bromazepam with respect to bioavailability after rectal administration. Thus, one of the major reasons why low bioavailability was observed after oral administration of bromazepam is considered to be degradation of the drug by the intestinal microflora.

On a thin layer chromatogram, the Rf values for bromazepam and its degradation product were 0.04 and 0.64, respectively, and no other spot was detected by this method. A major metabolite of bromazepam separated by TLC was identical with authentic 2-(2-amino-5-bromobenzoyl) pyridine on GC-MS analysis, as shown in Fig. 3. Thus, the degradation of bromazepam in intestinal microflora may be represented by the scheme shown in Chart 2.

Reversible hydrolytic reactions of 1,4-benzodiazepines in acidic solutions at body
temperature have been studied.\textsuperscript{8-10} After oral administration of 1,4-benzodiazepines, the open-ring compounds would be produced from the parent closed-ring compounds. When the open-ring compounds enter the intestine, they are expected to revert to the parent 1,4-benzodiazepines due to the higher pH value of the medium. Therefore, these reversible reactions of most 1,4-benzodiazepines may not influence their oral bioavailability.\textsuperscript{11} However, bromazepam was slowly but irreversibly degraded in the acidic media at 37 \degree C and decomposed by a two-step sequential reaction to 2-(2-amino-5-bromobenzoyl) pyridine and glycine.\textsuperscript{12} Thus, the reason why lower bioavailability of bromazepam was obtained after oral administration than after rectal administration may be considered to be the degradation of bromazepam both by acid in the stomach and by intestinal microflora.

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\textbf{References}