Use of the conjugate of disulphated ursodeoxycholic acid with \( p \)-aminobenzoic acid for the detection of intestinal bacteria

M Takahashi, T Konishi, Y Maeda, Y Matsugu, F Akazawa, T Eto, M Okajima, K Uchida, Y Masaoka, K Okada

Abstract

The disulphate ester of ursodeoxycholyl-\( p \)-aminobenzoic acid (PABA-UDCA) was synthesised and compared with PABA-UDCA for its use in detection of intestinal bacteria. This compound, PABA-UDCA disulphate, had characters in common with PABA-UDCA in that it was deconjugated by choliclygic acid hydrolase to release free PABA and bacteria that split glycocholic acid deconjugated PABA-UDCA disulphate. Further, in rat experiments urinary excretions of PABA were measured for six hours after oral administration of 15 mg PABA-UDCA disulphate. Ten control rats excreted (mean (SE) 188-2 (13-6) \( \mu \)g of PABA; 10 rats with an intestinal stagnant loop excreted more (530-1 (30-1) \( \mu \)g; \( p<0.001 \)); whereas 10 rats in each of three groups pretreated by oral administration of various antibiotics excreted less (polymixin B + tinidazole, 4-9 (1-6) \( \mu \)g; kanamycin, 31-0 (4-7) \( \mu \)g; clindamycin 40-9 (5-5) \( \mu \)g; \( p<0.001 \)).

By contrast with PABA-UDCA, PABA-UDCA disulphate was not actively absorbed from any part of the small intestine in everted gut sac experiments, and showed poor recovery from bile after its intraleral installation in rats. This indicated that PABA-UDCA disulphate is a single pass type substance in the gut and its oral administration test reflects the sum of the activities of bacteria in the small intestine and colon. The disulphate was easily soluble in water and this allowed its application in an in vitro test involving PABA-UDCA disulphate incubation with intraperitoneal pus (PABA-UDCA disulphate incubation test) from patients with peritonitis. This test was carried out on six patients with peritonitis, and the severity of bacterial peritonitis was expressed quantitatively. From the results obtained PABA-UDCA disulphate was considered a good material to detect intestinal bacteria.

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To evaluate intestinal bacteria, clinical application of breath tests with \( ^{14}C \) labelled glycocholate, \( ^{14}C \)-D-xylose, glucose-\( H_2 \), or lactulose \( H_2 \) has been proposed.\(^8\) These tests have flaws, however, because they need a radioisotopic material or special, expensive equipment for measurement. To solve these problems, we reported the use of a conjugate of ursodeoxycholic acid with \( p \)-aminobenzoic acid (PABA-UDCA) for the evaluation of intestinal microflora.\(^9\)

In this article we report studies on the use of a disulphated derivative of PABA-UDCA for the detection of intestinal bacteria.

Materials and methods

SYNTHESIS OF THE PABA CONJUGATE OF UDCA DISULPHATE

Synthesis of PABA-UDCA was by the method described previously.\(^4\) The disulphate ester of PABA-UDCA was prepared by a modification of the method of Goto and associates.\(^9\) To a solution of PABA-UDCA (1·0 g) in anhydrous pyridine (10 ml) was added chlorosulphonic acid (1·0 ml) in anhydrous pyridine (10 ml) with ice cooling and the solution was then heated overnight at 50°C. The resulting solution was poured into ice water, acidified with concentrated HCl, and extracted with \( n \)-butanol. The organic layer was washed with H\(_2\)O, dried over anhydrous Na\(_2\)SO\(_4\), filtered and the filtrate was concentrated in vacuo. The residue was chromatographed on a silica gel column with ethylammonium hydroxide (9:1 v/v). The appropriate fractions were collected and evaporated and the residue was crystallised from methanol-ethyl acetate to give 460 mg of the ammonium salt of PABA-UDCA disulphate (melting point 177–183°C); IRv \( \text{cm}^{-1} \): 2920, 2860, 1660, 1525; NMR (\( d_6 \), DMSO, \( \delta \)): 0·61 (3 \( H \), s), 0·89 (3 \( H \), s), 0·94 (3 \( H \), d, \( J=6 \)), 3·94~4·2 (2 \( H \), m), 7·70 (2 \( H \), d, \( J=9 \)), 7·87 (2 \( H \), d, \( J=9 \)), 10·18 (1 \( H \), s)). The ammonium salt was dissolved in \( H_2O \), adjusted to pH 9·5 with NaOH, and passed through a column of ion exchange resin (Diaion HP 20, Mitsubishi Chemical Industries, Japan) to give the sodium salt of PABA-UDCA disulphate, which was used in the reported experiments. Figure 1 shows the chemical structures of PABA-UDCA disulphate and PABA-UDCA. The RFs of PABA-UDCA disulphate and its related compounds on thin layer chromatography (Kieselgel 60 F\(_{254}\) plates, Merck) with a solvent system of benzene: dichloro: methanol:acetic acid (15:5:3:2, v/v) were: UDCA, 0·91; PABA-UDCA, 0·87; PABA-UDCA disulphate, 0·52.

IN VITRO HYDROLYSIS OF PABA-UDCA DISULPHATE WITH CHOLYLGLYCINE HYDROLASE

Incubation of PABA-UDCA disulphate with choliclyglycine hydrolase was carried out in accordance with the method described by Nair.\(^11\) Solutions with six different concentrations of PABA-UDCA disulphate were studied (A, B, C, D, E, and F respectively contained 2·2 mg,
1.1 mg, 0.55 mg, 0.27 mg, 0.14 mg, and 0.07 mg of PABA-UDCA disulphate in each 2 ml of reaction mixture (1.489 mM, 0.744 mM, 0.372 mM, 0.186 mM, 0.093 mM, and 0.047 mM)). The amount of PABA released was estimated with an assay system for urinary PABA (Eisai Co, Tokyo, Japan).12

INCUBATION OF PABA-UDCA DISULPHATE WITH PANCREATIC ENZYMES AND HOMOGENATES OF INTESTINAL MUCOSA FROM RATS

These experiments were carried out by the method reported by Huijgebaert and Hofmann.13

CULTURE OF PABA-UDCA DISULPHATE WITH AEROBIC AND ANAEROBIC INTESTINAL BACTERIA

This study was carried out as we described previously.3

ADMINISTRATION OF PABA-UDCA DISULPHATE TO RATS WITH DIFFERENT INTESTINAL BACTERIA

An in vivo experiment was carried out with male Sprague-Dawley rats (weight range 200–300 g) as we previously reported.4 Rats were divided into a control group (group A), an antibiotic pretreated group (group B), and a bacterial overgrowth group (group C). Group B was further divided into three subgroups in accordance with the prescription and dose of antibiotics, as: group B1, 30,000 units polymixin B+20 mg tinidazole; group B2, 50 mg kanamycin; group B3, 10 mg clindamycin. Each group and subgroup consisted of 10 rats. Bacterial overgrowth rats were prepared in the same way as described previously.4 After oral administration of 15.0 mg PABA-UDCA disulphate to each rat, the amounts of PABA excreted in a six hour urine sample were determined.

EVERTED GUT SAC EXPERIMENTS

Whether PABA-UDCA disulphate or PABA-UDCA was actively absorbed from the small intestine was studied with an everted rat gut sac. Male Sprague-Dawley rats weighing about 250 g were fasted for 24 hours and killed by decapitation. Laparotomy was performed, and the intestine was flushed with 100 ml of ice cold 0.9% NaCl solution. The entire small intestine was removed, and divided equally into four segments, (segments of jejunum I, jejunum II, ileum I, and ileum II from proximal to distal). The everted gut sac was prepared with 10 cm of small intestine from each segment in accordance with the method of Wilson and Wiseman.14 The sacs were filled with a modified Krebs-Ringer bicarbonate solution (pH 7.4) containing 0.1 mM PABA-UDCA disulphate or PABA-UDCA. The composition of the Krebs-Ringer solution was: 128 mM NaCl, 1.2 mM CaCl2, 0.7 mM MgSO4, 5.1 mM KCl, 1.3 mM KH2PO4, 25.6 mM NaHCO3, and 9 mM glucose. The gut sacs were placed in incubation flasks containing 30 ml of a similar solution to that of the serosal compartment. Under gentle oxygenation (95% O2-5% CO2), all flasks were incubated at 37°C for one hour. At the end of the incubation period, PABA-UDCA disulphate or PABA-UDCA concentration in the serosal and mucosal compartments was measured by the method of Bratton and Marshall after hydrolysis of PABA-UDCA disulphate or PABA-UDCA with HCl.15

BILIARY RECOVERY OF PABA-UDCA AND ITS DISULPHATE IN RATS

Male Sprague-Dawley rats (weight range 200–300 g) were fasted overnight before operation. Laparotomy was carried out under ether anesthesia and cannulation of the bile duct was performed for continuous bile collection. At the midpoint of the ileum, 0.020 mmol of PABA-UDCA disulphate (15.0 mg) or PABA-UDCA (10.0 mg) in a slightly basic (pH 7.5) aqueous solution was injected into the lumen, and bile was collected for three hours. Biliary recovery of the compounds was calculated by subtracting the

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Operative procedure</th>
<th>Total volume of intraperitoneal pus or fluid (ml)</th>
<th>Result of PABA-UDCA disulphate incubation test*</th>
<th>Bacterial culture examination of the sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Perforation of duodenal ulcer</td>
<td>Gastrectomy</td>
<td>90</td>
<td>0 µg</td>
<td>Negative</td>
</tr>
<tr>
<td>B</td>
<td>Ileocecal abscess due to appendicitis</td>
<td>Appendectomy</td>
<td>10</td>
<td>2-45 µg</td>
<td>Negative</td>
</tr>
<tr>
<td>C</td>
<td>Perforation of jejunum</td>
<td>Partial resection of ileum I</td>
<td>1000</td>
<td>3-57 µg</td>
<td>Clostridium perfringens</td>
</tr>
<tr>
<td>D</td>
<td>Perforative abscess due to ischemic colitis of the sigmoid colon</td>
<td>Sigmoidectomy</td>
<td>20</td>
<td>1-83 µg</td>
<td>Enterobacter aerogenes</td>
</tr>
<tr>
<td>E</td>
<td>Massive necrosis of the jejunum and ileum (thrombosis of superior mesenteric artery)</td>
<td>Massive resection of the small intestine</td>
<td>940</td>
<td>11-11 µg</td>
<td>Streptococcus intermedius</td>
</tr>
<tr>
<td>F</td>
<td>Radiation ileitis</td>
<td>Resection of ileum</td>
<td>800</td>
<td>20.37 µg</td>
<td>Bacteroides fragilis</td>
</tr>
</tbody>
</table>

*Values express the amounts of PABA released by 30 minute incubation of PABA-UDCA disulphate with 1 ml of intraperitoneal pus or fluid.
amounts of biliary PABA measured without HCl hydrolysis from those with hydrolysis.

INSTILLATION OF PABA INTO THE COLON IN HUMANS
To investigate whether PABA was absorbed from the colon, an instillation was carried out in three patients. One of the patients (patient 1) had an obstructing tumour in the transverse colon with a loop colostomy in the caecum and two (patients 2 and 3) had obstructing tumours in the sigmoid colon with loop colostomy in the transverse colon. PABA (100 mg) dissolved in 20 ml of saline solution was administered to the anal side of the colon through the colostomy, and urinary excretion of PABA was measured for six hours after instillation. Each one hour excretion of PABA after instillation was obtained by subtracting background PABA from a one hour urine sample before instillation.

INCUBATION OF PABA-UDCA DISULPHATE WITH INTRAPERITONEAL PUS OR FLUID FROM PATIENTS WITH PERITONITIS
To examine the magnitude of contamination of the peritoneal cavity by intestinal bacteria, an incubation of PABA-UDCA disulphate with intraperitoneal pus or fluid was carried out in six patients with localised or diffuse peritonitis

STATISTICAL ANALYSIS
Results are expressed as mean (SE). Statistical comparisons were made with Student’s t test for unpaired samples. A p value <0·01 was considered significant.

Results
HYDROLYSIS OF PABA-UDCA DISULPHATE BY CHOLYLGLYCINE HYDROLASE
Figure 2 shows the time course of enzymatic hydrolysis of PABA-UDCA disulphate at six different concentrations of substrate. The compound was efficiently hydrolysed by cholylglycine hydrolase. The Km value determined from the Lineweaver-Burk plot was 0·23 mM.

HYDROLYSIS OF PABA-UDCA DISULPHATE BY PANCREATIC ENZYMES AND RODENT INTESTINAL MUCOSAL HOMOGENATES
PABA-UDCA disulphate was completely resistant to pancreatic enzymes such as pancreatic, carboxypeptidases A and B, α-chymotrypsin, and trypsin. Similarly it was not hydrolysed by mucosal homogenates from the small intestine of the rat.
BACTERIAL DECONJUGATION

Tables II and III show bacterial deconjugation data. Of the aerobic bacteria, only Enterococcus faecalis, Lactobacillus acidophilus, Proteus mirabilis, and Staphylococcus epidermidis deconjugated PABA-UDCA disulphate.

By contrast, many anaerobic intestinal bacteria deconjugated this compound. These included Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides vulgatus, Bifidobacterium adolescentis, Bifidobacterium longum, Clostridium perfringens, Eubacterium aerofaciens, and Fusobacterium varium.

ADMINISTRATION OF PABA-UDCA DISULPHATE IN RATS

Figure 3 shows the results of the urinary excretion of PABA for six hours after dosing for all groups of rats. No adverse effects were noted in the rats and no deaths occurred. The control group of rats excreted (mean (SE)) 188 ± 2 (13-6) μg of PABA into urine. Significant (p<0.001) suppressions of urinary PABA excretion were found in rats pretreated with antibiotics compared with the control group (B1, 4-9 (1-6) μg; B2, 31-0 (4-7) μg; B3, 40-9 (5-5) μg). By contrast, rats with intestinal bacterial overgrowth had significantly greater excretion of PABA than the controls (530 ± 1 (30-1) μg (p<0.001)).

INTESTINAL ABSORPTION OF PABA-UDCA AND ITS DISULPHATE

Figure 4 shows the results of the active transport experiment, indicating that PABA-UDCA was actively absorbed from the distal side of the ileum whereas its disulphate was not absorbed from any part of the small intestine. In vivo, considerable PABA-UDCA was recovered from bile (32-6 (1-3)% (n=3)), whereas recovery of PABA-UDCA disulphate was poor (1-8 (0-2)% (n=3)), after intraligal instillation.

ABSORPTION OF PABA FROM THE COLON IN HUMANS

Figure 5 shows urinary recovery of PABA after intracolonic instillation of PABA in human subjects. The maximal urinary excretion of PABA was found three or four hours after PABA instillation. Total recoveries of PABA during six hours after instillation were 37-7%, 49-5%, and 38-5% in patients 1, 2, and 3 respectively.

PABA-UDCA DISULPHATE INCUBATION TEST IN PATIENTS WITH PERITONITIS

PABA-UDCA disulphate was easily soluble in assay solution with pH 5-6 sodium acetate buffer, whereas the maximal solubility of PABA-
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UDCA in that solution was 1.2 µg/ml. The incubation data were obtained within 90 minutes after starting the test. Table I shows the results.

These sulphate esters of bile acids are usually less toxic than their unesterified parent substances and are eliminated more rapidly through faeces and urine so that the organism can be efficiently detoxified in the case of cholestasis. Lack et al. and Walker et al. reported that bile and sulphates were absorbed from the intestine to a lesser extent than their unesterified parent substances. Likewise the disulphate ester of PABA-UDCA was not actively absorbed from any part of the small intestine, and was recovered from bile in only small amounts in our experiment.

Eysen et al., Huighebaert et al., and Pacini et al. previously confirmed the absence of bacterial desulphating activity on 7-monosulphate esters of bile acids but the presence of such desulphating activity on 3-monosulphate esters in cultures of faeces from humans, rats, and mice. Consequently, even if the disulphate ester of PABA-UDCA is desulphated at the 3α position by bacterial action after oral administration the 7β-monosulphate ester of PABA-UDCA formed can be resistant to bacterial transformation resulting in no absorption from the gut and rapid excretion into faeces. Therefore PABA-UDCA disulphate can be a single pass type of substance in the gut. Contrary to our expectations, PABA that was instilled into the human colon was recovered well from urine. This means that there is a good absorption of PABA from the colon. For these reasons, we can deduce that PABA-UDCA disulphate is a single pass type of compound in the gut for the evaluation of intestinal microflora, and the administration test results reflect the sum of activities of bacteria in the small intestine and colon. Practically, the oral administration test with PABA-UDCA disulphate makes possible the evaluation of colonic bacteria because most intestinal bacteria is present in the colon. Also, the test may be useful for monitoring the effects of antibiotics on the gastrointestinal bacteria.

The third difference is in the solubility of the compounds in water. PABA-UDCA was practically insoluble in the assay solution containing 2-mercaptoethanol, EDTA, and pH 5–6 sodium acetate buffer. It was rendered easily soluble in it, however, by the introduction of sulphate groups on the 3α and 7β hydroxyl groups of PABA-UDCA. This increase in water solubility made possible an in vitro incubation test with intraperitoneal pus or fluid from patients with peritonitis. Positive results in this incubation test indicate the intraperitoneal presence of cholyglycine hydrolase, namely intraperitoneal leakage of intestinal bacteria. Data obtained from the test reflect the magnitude of intraperitoneal contamination by intestinal bacteria. Considered from the standpoint of bacterial peritonitis and prediction of prognosis, it is important to know how much the patients with peritonitis are contaminated by intestinal bacteria. We consider that this PABA-UDCA disulphate incubation test makes it possible to express the severity of bacterial peritonitis quantitatively within 90 minutes.

Intestinal bacteria are still difficult to assess due to the complexity and expense of measurement of intestinal microorganisms. The PABA-UDCA disulphate described in this article might...
be a material to improve this situation. Further studies should be done on humans.

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