Usefulness of Breath Test for Evaluating Pancreatic Exocrine Function Using N-Benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine Sodium in Non-invasive and Conscious Rats

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\(N\)-Benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine sodium is a dipeptide used for evaluating pancreatic exocrine function. The method of evaluation, however, is not yet satisfactory, especially in experimental animals. The relation between diabetes and pancreatic exocrine function also is not clear. Therefore, this study sought to establish a method for evaluating pancreatic exocrine function and to validate the method by determining non-invasively the effect of alloxan-induced diabetes in conscious rats. After fasting, rats were orally administered Racol containing \(N\)-benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine sodium or 1-\(^{13}\)C-L-alanine and housed in desiccators. The expired air in the desiccator was collected in a breath-sampling bag using a tube and aspiration pump, and the level of \(^{13}\)CO\(_2\) in this air was measured using an infrared spectrometer at appropriate intervals over a 120 min period. The rate of \(^{13}\)CO\(_2\) excretion increased, peaked and then decreased in a dose-dependent manner. The maximum concentration and area under the curve of \(^{13}\)CO\(_2\) excretion significantly and positively correlated with the doses of \(N\)-benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine sodium. In the rats made diabetes by the administration of alloxan, the level of expired \(^{13}\)CO\(_2\) air changed at significantly lower levels as compared with that of the control rats on day 3, although the level of expired \(^{13}\)CO\(_2\) air from 1-\(^{13}\)C-L-alanine was also markedly lower than that of the control rats. These results showed that pancreatic exocrine function can be evaluated using this breath test system and that alloxan-induced diabetes affects this function.

Key words \(N\)-benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine; pancreatic exocrine function; breath test; alloxan; diabetes

An exocrine pancreatic function test using secretin-cholecystokinin or caerulein is considered the best test possible because of its high sensitivity and specificity.\(^1,2\) However, this test is complex, time consuming, unpleasant for the patient and not feasible in some cases. Therefore, many alternative methods have been developed, such as the \(p\)-aminobenzozoic acid and fluorescein dilaurate tests, serum trypsin and faecal chymotrypsin concentrations,\(^3\) and enzyme linked immunosorbent assay for elastase in feces.\(^4\) The ideal exocrine pancreatic function test, however, would be sensitive, specific, easy to perform, non-invasive and inexpensive.

On the other hand, \(^{13}\)C-breath tests have been developed as a nonradioactive alternative method. Ghoos and colleagues\(^5\) developed a breath test in 1993 to monitor gastric emptying using \(^{13}\)C-labelled acetic acid. In 2007, Kohno and colleagues\(^6\) synthesized \(^{13}\)C-dipeptide, \(N\)-benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine sodium, and evaluated pancreatic exocrine function by administering \(N\)-benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine sodium dissolved in distilled water to anesthetized or conscious rats. However, it is important to perform a breath test non-invasively while the animal is in a conscious and physiologically functioning state. Nutrients in the test meal also affect the pancreatic exocrine function. Recently, we developed a simple breath test system for monitoring gastric emptying non-invasively using Racol (an enteral nutritional formula) containing \(^{13}\)C-labeled acetic acid in conscious rats.\(^8\) Racol has been used in clinics to investigate the gastric emptying. This study thus aimed to evaluate the pancreatic exocrine function using Racol containing \(N\)-benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine sodium and a recently developed simple and non-invasive breath test system.

On the other hand, pancreatic exocrine function is known to be low in patients of pancreatitis. However, the effect of diabetes on the pancreatic exocrine function has not been clarified particularly in experimental animals. To evaluate the usefulness of the present method, this function was thus investigated in the alloxan-induced diabetic rats.

In diabetic patients, the gastric emptying rate is known to be delayed. To determine the effect of pancreatic insufficiency resulting from diabetic mellitus due to the slower gastric emptying rate, we further tested the effect of this condition on the change of expired air from 1-\(^{13}\)C-L-alanine which is digested by pancreatic enzymes from \(N\)-benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine sodium.

MATERIALS AND METHODS

The following animal studies were performed according to “Guiding Principles for the Care and Use of Laboratory Animals” approved by The Japanese Pharmacological Society.

Animals Male Fisher-344 rats (200—250 g) were purchased from SLC (Shizuoka, Japan), and kept for 1 week before experiments in a room where the temperature and humidity were kept at 21±2 °C and 55±15%, respectively. The light and dark cycle was 12 h, and light period was from 7:00 to 19:00. Rats were fasted in mesh cages for one night to prevent coprophagy before each experiment, but had free access to drinking water.

Breath Test Using \(N\)-Benzoyl-L-tyrosyl-1-\(^{13}\)C-L-alanine Sodium or 1-\(^{13}\)C-L-Alanine in Conscious Rats A breath test was performed according to our reported method.\(^7\) In brief, this system is composed of an animal chamber (desiccator, 2000 ml), pump (Masterflex L/S, Cole-Palmer Inst. Co., U.S.A.) and breath sampling bag (Ohtsuka Pharmaceutical Co. Ltd., Tokyo, Japan). Collected \(^{13}\)CO\(_2\) air was measured with UBiT IR-300 and UBiT-AS10 (Ohtsuka Pharma-
Rats were placed in the chamber just after the oral administration of a sample containing \(N\)-benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium or \(1^{-13}\)C-L-alanine sodium dissolved in Racol (enteral nutritional formula) and administered at 2.5 ml/kg. Doses of \(1^{-13}\)C-L-alanine sodium were 10, 30 and 100 mg/kg. \(1^{-13}\)C-L-Alanine and alloxan were purchased from Isotec (Miamisburg, Ohio, U.S.A.) and Sigma (Tokyo), respectively. \(1^{-13}\)C-L-alanine sodium was 6.3 mg/kg [the same molar as \(^{13}\)CO\(_2\) Air from \(1^{-13}\)C-L-Alanine was dissolved in Racol (enteral nutritional formula) and administered orally at 2.5 ml/kg and expired \(^{13}\)CO\(_2\) air was monitored for 120 min. The same treatment was performed thereafter (Fig. 1). Ventilation volume was 150 ml/min.

**Evaluation of Breath Test** The measured values were presented as the \(\Delta{^{13}}\)CO\(_2\) (‰). The maximum concentration (\(C_{\text{max}}\); ‰), the time taken to reach the maximum concentration (\(T_{\text{max}}\); min) and the area under the curve (\(AUC_{120\text{min}}\); ‰·min) were calculated using the \(\Delta{^{13}}\)CO\(_2\) values. \(C_{\text{max}}\) and \(AUC_{120\text{min}}\) reflect the absorption of digested materials by protease.

**Dose-Dependency of \(N\)-Benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine Sodium** \(N\)-Benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium was dissolved in Racol (enteral nutritional formula) and administered in a volume of 2.5 ml/kg. Doses of \(N\)-benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium were 10, 30 and 100 mg/kg.

**Induction of Alloxan-Induced Diabetes** Diabetes was induced by the intravenous administration of alloxan dissolved in saline at a dose of 40 mg/kg (2.5 ml/kg) according to the methods reported previously with slight modifications. In the control rats, saline was administered instead of alloxan.

Blood glucose levels were measured with a Glucometer DEX (Bayer Health Care, U.S.A.) on Day 2 after alloxan administration.

**Effect of Alloxan-Induced Diabetes on the Expired \(^{13}\)CO\(_2\) Air from \(N\)-Benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine Sodium** Two days after alloxan administration, rats were fasted for one night, and a breath test was performed by the method described above. In brief, 30 mg/kg of \(N\)-benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium dissolved in Racol was administered orally at 2.5 ml/kg and expired \(^{13}\)CO\(_2\) air was monitored for 120 min. The same treatment was performed in the control rats.

**Effect of Alloxan-Induced Diabetes on the Expired \(^{13}\)CO\(_2\) Air from \(1^{-13}\)C-L-Alanine** \(1^{-13}\)C-L-Alanine was dissolved in Racol and administered at 2.5 ml/kg. The dose of \(1^{-13}\)C-L-alanine sodium was 6.3 mg/kg [the same molar as \(N\)-benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium (30 mg/kg)].

**Agents** \(N\)-Benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium and Racol were purchased from Tokyo Gas Chemical Co., Ltd. (Tokyo, Japan) and Ohtsuka Pharmaceutical Co., Ltd., respectively. \(1^{-13}\)C-L-Alanine and alloxan were purchased from Isotec (Miamisburg, Ohio, U.S.A.) and Sigma (Tokyo), respectively.

**Statistical Analysis** Results were represented as the mean±S.E.M. of animals used. Statistical analyses were performed by a Student’s \(t\)-test and values of \(p<0.05\) were regarded as significant.

**RESULTS**

**Dose-Dependency of \(N\)-Benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine Sodium** Expired \(^{13}\)CO\(_2\) air increased with time after ingestion in a dose-dependent manner, peaked and decreased thereafter (Fig. 1).

\(C_{\text{max}}\) values increased in a dose-dependent manner (Table 1). A significant positive correlation was observed between doses of \(N\)-benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium and \(C_{\text{max}}\) values (Fig. 2A) \((p<0.05)\). The value of \(AUC_{120\text{min}}\) also increased in a dose-dependent manner (Table 1). A significant positive correlation was observed between doses of \(N\)-benzoyl-L-tyrosyl-\(^{13}\)C-L-alanine sodium and \(AUC_{120\text{min}}\) values (Fig. 2B) \((p<0.05)\).
The values represent the mean±S.E. (n=4). *,** Significant difference from the control rats (p<0.05, 0.01).

Table 2. Effect of Alloxan-Induced Diabetes (Diabetes) on the Pharmacokinetic Parameters of N-Benzoyl-L-tyrosyl-1-13C-L-alanine Sodium in Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (%)</td>
<td>60.9±1.3</td>
<td>50.9±4.3*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>22.5±1.4</td>
<td>28.8±2.4*</td>
</tr>
<tr>
<td>$AUC_{120\text{min}}$ (‰·min)</td>
<td>4251±198</td>
<td>3714±210</td>
</tr>
</tbody>
</table>

Values represent the mean±S.E. (n=4). * shows a tendency to decrease or delay (p<0.10).

$T_{\text{max}}$ values were delayed as the dosage was increased (Table 1).

Effect of Alloxan-Induced Diabetes on the Expired 13CO2 Air from N-Benzoyl-L-tyrosyl-L-13C-L-alanine Sodium

On Day 2 after diabetes induction, blood glucose levels were 126±6.0 and 415±11.1 mg/dl in the control group and diabetes-induced group, respectively. A significant difference was observed between the two groups (p<0.01). The effect of alloxan-induced diabetes is shown in Fig. 3. In the control rats, expired 13CO2 air increased with time after ingestion, peaked at 22.5 min and decreased thereafter. The diabetic rats, expired 13CO2 air changed at markedly lower levels as compared with control rats.

Effect of Alloxan-Induced Diabetes on the Expired 13CO2 Air from L-13C-L-Alanine

On Day 2 after diabetes induction, blood glucose levels were 129±4.2 and 428±3.6 mg/dl in the control group and diabetes-induced group, respectively. A significant difference was observed between the two groups (p<0.01).

The effect of alloxan-induced diabetes on the expired 13CO2 air form L-13C-L-alanine sodium is shown in Fig. 4. In the control rats, this expired air increased, peaked and decreased over time. In the diabetic rats, this expired 13CO2 air changed at markedly lower levels as compared with control rats, although significant differences were not observed between the two groups.

$C_{\text{max}}$ and $AUC_{120\text{min}}$ values in diabetic rats are shown in Table 3 and are not significantly different from those in the control rats. However, $T_{\text{max}}$ value in diabetic rats showed a tendency to be delayed (p<0.10).

DISCUSSION

It is assumed that orally administered N-benzoyl-L-tyrosyl-1-13C-L-alanine sodium is evacuated to the duodenum, where it is cleaved by one of the pancreatic proteases, carboxypeptidase, thereby releasing 13C-alanine. 13C-Alanine is then rapidly absorbed from the intestinal mucosa and metabolized in the liver. Thereafter, 13CO2 air is exhaled from the mouth. Kohno and colleagues evaluated the pancreatic exocrine function in anesthetized or conscious rats after oral administration of N-benzoyl-L-tyrosyl-1-13C-L-alanine sodium. However, it is important to perform breath test non-invasively while the rat is in a physiological state. Therefore, we used our recently developed breath test system using conscious rats. In the present study, exhaled 13CO2 air increased with time, then peaked and decreased after the administration of N-benzoyl-L-tyrosyl-1-13C-L-alanine sodium. Moreover, a significant positive correlation was observed between the doses of N-benzoyl-L-tyrosyl-1-13C-L-alanine sodium and $C_{\text{max}}$ or $AUC_{120\text{min}}$ values. These results show that the N-benzoyl-L-tyrosyl-1-13C-L-alanine sodium is digested by the protease secreted from the pancreas, and that the monitoring of the expired 13CO2 air from this reflects the pancreatic exocrine function.

Lembcke and colleagues reported that in patients with pancreatic disease, the 13C-Hiolein breath test reflects impaired lipase output indicating decompensated lipolysis. The breath test using 13C-Hiolein is a convenient alternative to faecal fat analysis. Lüöser and colleagues reported that the 13C-mixed triglyceride breath test reflects severe exocrine pancreatic insufficiency (steatorrhea) but has limited sensi-
tivity for the detection of mild cases. Ishii and colleagues reported that the N-benzoyl-L-tyrosyl-1-13C-L-alanine breath test can quickly, safely and non-invasively diagnose exocrine pancreatic dysfunction in a clinical study and found that expired 13CO2 values at 10—60 min for this test were significantly lower in chronic pancreatitis patients than in normal controls. In general, pancreatic exocrine function is known to be low in pancreatitis patients.

On the other hand, diabetes mellitus is also known to be associated with pancreatic atrophy and compromised digestion of carbohydrates as a result of exocrine pancreatic insufficiency and lower alpha-amylase synthesis and secretion. Kahara and colleagues reported that total protein and amylase secretion was insufficient in alloxan-induced diabetic rats. Alvarez and López reported that total protein and amylase secretion decreased markedly in the alloxan-induced diabetic rabbits. This may support our present findings that pancreatic exocrine function was insufficient in alloxan-induced diabetic rats. These results confirm that this method is useful to evaluate pancreatic exocrine function.

In conclusion, pancreatic exocrine function was evaluated using N-benzoyl-L-tyrosyl-1-13C-L-alanine sodium and our recently developed non-invasive breath test system in conscious rats. Moreover, insufficiency of the pancreatic exocrine function was observed in alloxan-induced diabetic rats. These results confirm that this method is useful to evaluate pancreatic exocrine function.

REFERENCES