RESEARCH PAPER

Proof-of-concept study on the suitability of $^{13}$C-urea as a marker substance for assessment of in vivo behaviour of oral colon-targeted dosage forms

RCA Schellekens$^{1,2,*}$, GG Olsder$^{1,*}$, SMCH Langenberg$^3$, T Boer$^3$, HJ Woerdenbag$^2$, HW Frijlink$^2$, JGW Kosterink$^1$ and F Stellaard$^3$

$^1$Department of Clinical and Hospital Pharmacy, University Medical Center Groningen, Groningen, The Netherlands, $^2$Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Groningen, The Netherlands, and $^3$Laboratory of Medicine, Center for Liver, Digestive and Metabolic Diseases, University Medical Center Groningen, Groningen, The Netherlands

Background and purpose: $^{13}$C-urea may be a suitable marker to assess the in vivo fate of colon-targeted dosage forms given by mouth. We postulated that release in the colon (urease-rich segment) of $^{13}$C-urea from colon-targeted capsules would lead to fermentation of $^{13}$C-urea by bacterial ureases into $^{13}$CO$_2$. Subsequent absorption into the blood and circulation would lead to detectable $^{13}$C (as $^{13}$CO$_2$) in breath. If, however, release of $^{13}$C-urea occurred in the small intestine (urease-poor segment), we expected detectable $^{13}$C (as $^{13}$C-urea) in blood but no breath $^{13}$C (as $^{13}$CO$_2$). The differential kinetics of $^{13}$C-urea could thus potentially describe both release kinetics and indicate the gastrointestinal segment of release.

Experimental approach: The in vivo study consisted of three experiments, during which the same group of four volunteers participated.

Key results: The kinetic model was internally valid. The appearance of $^{13}$C-in breath CO$_2$ ($F_{\text{fermented}}$) and the appearance of $^{13}$C in blood as $^{13}$C-urea ($F_{\text{not fermented}}$) show a high inverse correlation (Pearson’s $r = -0.981$, $P = 0.06$). The total recovery of $^{13}$C ($F_{\text{fermented}} + F_{\text{not fermented}}$) averaged 99%, indicating complete recovery of the administered $^{13}$C via breath and blood. $^{13}$CO$_2$ exhalation was observed in all subjects. This indicates that $^{13}$C-urea was available in urease-rich segments, such as the caecum or colon.

Conclusions and implications: In this proof-of-concept study, $^{13}$C-urea was able to provide information on both the release kinetics of a colon-targeted oral dosage form and the gastrointestinal segment where it was released.

Keywords: colon delivery; colon-targeting; stable isotope; $^{13}$C-urea; fermentation; intestinal metabolism; bioavailability; urea; OCTT

Abbreviations: GIT, gastrointestinal tract; H. pylori, Helicobacter pylori; LNA, Laboratory of Dutch Pharmacists; OCTT, oro-caecal transit time; Ph.Eur., European Pharmacopoeia; UDV, urea distribution volume; WFI, water for injections

Introduction

Drug delivery to the ileocaecal segment of the gastrointestinal tract (GIT) is considered beneficial in therapy of diseases affecting the colonic mucosa and for delivery of drugs which are inactivated in the upper gastrointestinal (GI) regions. Continuous efforts are being made on designing colon-specific delivery systems to accommodate different therapeutic objectives (Yang et al., 2002; Ibekwe et al., 2006a,b; Kumar and Mishra, 2008). Characterization of the in vivo release profile of oral drug delivery systems is generally performed by conventional pharmacokinetic assessment in combination with an imaging technology (Coupe et al., 1991). In earlier days, the transit of a dosage form through the GIT was visualized by using X-ray radiography with barium sulphate as a contrast substance (Lark-Horovitz, 1941; Efimova and Minina, 1969). Today, $\gamma$-scintigraphy as a molecular imaging technology is considered to be the state-of-the-art imaging technology (Digenis et al., 2000; Wilding et al., 2001; Damle et al., 2002; Willmann et al., 2008). While recognizing the advantages of the above-mentioned methods,
they both carry the disadvantage of exposing subjects to ionizing radiation. Sulfasalazine has also been used to qualitatively measure the small intestinal transit time (Sunesen et al., 2005). However, sulfasalazine is not a reliable marker for showing the fraction of the dose delivered in the colon because of its limited absorption from the small intestine and its enterohepatic circulation. Assessment of in vivo behaviour of oral drug products by pharmacokinetic assessment in combination with non-radioactive stable isotope technology is seldom performed, despite its interesting possibilities (Verbeke et al., 2005).

A marker substance for colon delivery assessment should ideally fulfil several requirements. First, the marker should be able to show whether release and/or uptake takes place in the colon or in other intestinal segments. Second, the marker or its relevant metabolite should have favourable kinetics such as fast and complete absorption, short distribution time and single compartment kinetics (small volume of distribution). Only in this case will the marker kinetics reflect the release kinetics of the dosage form. Furthermore, the safety of the marker, ease of sampling and reliable analysis are important issues. In view of these specifications, $^{13}$C-urea provides an interesting possibility.

We proposed that release of $^{13}$C-urea in the caecum or colon (urease-rich segment) from oral colon-targeted capsules would lead to fermentation of $^{13}$C-urea by bacterial urease into $^{13}$CO$_2$ (Lee et al., 2003; Urita et al., 2006). Subsequent absorption of $^{13}$CO$_2$ into the blood and circulation would lead to detectable $^{13}$C (as $^{13}$CO$_2$) in breath. If, however, release of $^{13}$C-urea already occurs in the small intestine (urease-poor segment), only detectable $^{13}$C (as $^{13}$C-urea) in the blood is expected, as the bioavailability of $^{13}$C-urea is near 100%. Consequently, no $^{13}$C (as $^{13}$CO$_2$) in breath will be detectable in the latter situation. This differential kinetics of $^{13}$C-urea (Figure 1)

![Figure 1](image-url)
could potentially describe both the kinetics of release and serve as an indicator for the GI segment of release.

In this study, we aim to give a proof of concept as to the suitability of $^{13}$C-urea as a marker substance for the assessment of in vivo behaviour of oral colon-targeted dosage forms.

**Methods**

**In vivo release testing**

Four apparently healthy volunteers (age 18–65 years) participated in the study. They had no history of GI diseases (e.g. colitis ulcerosa, Crohn’s disease, spastic colon, colon carcinoma, ileus, stoma, stomach and/or intestinal infection) or of GI surgery (with the exception of appendectomy). They had not taken antibiotics or medicines influencing GI transit time for at least 3 months.

Before participation, each subject was tested for the absence of *Helicobacter pylori* (H. pylori) using the licensed Pylobactell® test (Torbet Laboratories Limited, Norwich, UK). Subjects testing positive for *H. pylori* were excluded from the study. The study was approved by the ethical committee and registered in the European Trials Database (nr. 2006-002125-26).

The in vivo study consisted of three experiments, one with an uncoated capsule and two with coated capsules. In all experiments, the subjects were fasted on day 1 from 20:00 h on. Only water and tea (no sugar) were allowed. In the morning on day 2, they received the capsule together with 150 mL apple juice. After a predetermined period of 3 hours after capsule intake, a standardized meal (the subsequent meal) consisting of a double sandwich was consumed in order to control the oro-caecal transit time (Priebe et al., 2004; 2006; Schellekens et al., 2008). In the first experiment, the volunteers received an uncoated capsule containing 100 mg $^{13}$C-urea, aimed to give information on the bioavailability of $^{13}$C-urea after release in the stomach and/or proximal small intestine (urease-poor region). In the second experiment, a coated capsule containing 100 mg $^{13}$C-urea was administered, aimed to give information on the availability of $^{13}$C-urea or its metabolite $^{13}$CO$_2$ after release in the ileo-caecal intestinal parts (urease-rich regions). In the third experiment, a coated capsule containing 100 mg $^{13}$C-bicarbonate was administered, aimed to give information on the availability of $^{13}$CO$_2$ in the ileo-caecal parts. Breath and blood samples were collected according to a set time schedule for 24 h ($^{13}$C-urea capsules) or 9 h ($^{13}$C-bicarbonate capsule). Breath samples were collected by breathing through a straw into 10 mL Exetainer tubes (Labco Limited, Buckinghamshire, UK). Four millilitre blood samples were collected in heparin tubes (BD, Breda, The Netherlands). During each experiment, the subjects received a slow, peripheral infusion of sodium chloride solution (0.9%) in between the blood samplings. Breath samples were centrifuged and plasma was stored at $-20^\circ$C until analysis. From 0.5 mL, plasma endogenous bicarbonate was removed by acidification with acetic acid and subsequent evaporation under nitrogen (Rembacz et al., 2007). Then, urea was converted to CO$_2$ using the enzyme urease (1% urease in ethylenediaminetetraacetic acid (EDTA) buffer) (Kloppenburg et al., 1997). After incubation, phosphoric acid (1M) was added to convert dissolved $^{13}$C-bicarbonate in the solution to $^{13}$CO$_2$ in the vapour phase. Finally, the $^{13}$C/$^{12}$C isotope ratio was determined in CO$_2$ in the headspace using the same IRMS instrumentation. Urea concentrations in plasma were determined using the Roche Modular® analyser (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

**Calculation of the concentration of $^{13}$C-urea in plasma**

The $^{13}$C$_{CO_2}$ values obtained from the plasma samples were converted to atom percentage excess $^{13}$C by the equations described by Schellekens et al. (2008). The concentration of $^{13}$C-urea in a plasma sample at any time point can then be calculated by Equation (1):

$$\left( ^{13}\text{C} - \text{urea} \right)_{t} = \frac{\text{APE} \times ^{13}\text{C} \times \text{urea}_0}{100}$$

The concentration versus time graph is described by Equation (2):

$$\log\left( ^{13}\text{C} - \text{urea} \right)_{t} = \log\left( ^{13}\text{C} - \text{urea} \right)_{t=0} - a \times t$$

where $a$ is the slope of the curve.

Subsequently, several kinetic parameters were deduced, based on the assumption that $^{13}$C-urea kinetics may be described by a one-compartment model (Kloppenburg et al., 1997). The lag time (starting point of release) of coated capsules is defined as the time point at which the area under the curve (AUC) is 5% of the AUC at $t = 12$ h ($t_{95}$). Based on Equation (2), the intercept with the y-axis ($t = 0$ h) of the curve in the steady state phase represents the fictitious ($^{13}$C-urea)$_{\text{fict}}$ Value. According to conventional kinetics theory, the apparent urea distribution volume (UDV in L·kg$^{-1}$ Lean Body Mass (LBM)) is then calculated by Equation (3):

$$\text{UDV} = \frac{\text{Dose}\left( ^{13}\text{C} - \text{urea} \right)}{\left( ^{13}\text{C} - \text{urea} \right)_{t=0} \times \text{LBM}_{\text{subject}}}$$

Then, the elimination rate constant ($k_{\text{el}}$) is calculated by Equation (4), in which $a$ is the slope of the semi-logarithmic concentration versus time graph in the steady state phase.

$$k_{\text{el}} = 2.303 \times a$$

Subsequently, the half-life ($t_{1/2}$) is calculated by Equation (5):

$$t_{1/2} = \frac{0.693}{k_{\text{el}}}$$

The pulsatile release properties are reflected by the so-called pulse time, defined as the time period between the lag time ($t_{95}$) and $t_{\text{max}}$. The availability of $^{13}$C-urea in urease-poor segments is expressed by the not fermented fraction ($F_{\text{not fermented}}$).
The internal validity of the model was tested by plotting the AUC of $^{13}$C-urea from the coated capsule 5 h after the lag time is defined as the time point at which the cumPDR is 5% (Schellekens et al., 2008). The lag time is calculated by the time difference between the maximal obtainable recovery in breath of $^{13}$CO$_2$ absorbed from the ileal-colonic intestines. Therefore, the availability of $^{13}$C-urea absorbed from these segments is taken as one because urea conforms to the Rule of 5 (Lipinski et al., 1997).

**Calculation of the percentage dose recovered $^{13}$C in breath**

The calculations used to convert $^{12}$C$_{\text{ER}}$ values into the percentage dose recovered per hour (PDR h$^{-1}$) and the cumulative PDR (cumPDR) have been described before (Schellekens et al., 2008). The lag time is defined as the time point at which the cumPDR is 5% ($t_{5\%}$) of the cumPDR at $t = 12$ h. The cumPDR at 12 h is calculated by linear interpolation of the nearest measurements ($^{13}$C-urea coated capsule) or linear extrapolation ($^{13}$C-bicarbonate-coated capsule) of the nearest measurements. The time point corresponding to the 5% value is also calculated by linear interpolation of the nearest measurements. The cumPDR at 12 h obtained applying $^{13}$C-bicarbonate as the tracer compound is considered to be the maximal obtainable recovery in breath of $^{13}$C$_2$O absorbed from the ileal-colonic intestines. Therefore, the availability of $^{13}$C-urea in urease-rich segments ($F_{\text{fermented}}$) is calculated relative to this maximum and thus, corrected for CO$_2$ retention according Equation (7). The pulse time is calculated by the time difference between the $t_{\text{max}}$ and $t_{5\%}$.

$$F_{\text{fermented}} = \frac{\text{cumPDR}_{12\,\text{h}}(^{13}\text{C-urea})}{\text{cumPDR}_{12\,\text{h}}(^{13}\text{C-bicarbonate})} \times 100\%$$

**Validity of the model**

The internal validity of the model was tested by plotting $F_{\text{fermented}}$ versus $F_{\text{not fermented}}$ and by calculating the total recovery of $^{13}$C ($F_{\text{total}} = F_{\text{fermented}} + F_{\text{not fermented}}$). The model was considered valid when $F_{\text{fermented}}$ and $F_{\text{not fermented}}$ were inversely related and $F_{\text{total}}$ was close to 100%.

**Statistical procedures**

The results were analyzed by descriptive statistics. Furthermore, we used the paired $t$-test to compare the behaviour of the two types of capsules (two-tailed, $\alpha = 0.05$). We also present 95% confidence intervals (95%CI) for the differences. Averages are presented with their coefficient of variation (CV). The correlation between the two dependent variables $F_{\text{fermented}}$ and $F_{\text{not fermented}}$ was determined by the Pearson product moment correlation coefficient (Pearson’s r).

**Results**

All subjects were tested negative for the presence of H. pylori, meaning that the $^{13}$C$_{\text{ER}}$ abundance in breath CO$_2$ 30 min after administration of $^{13}$C-urea as an oral solution was less than 5% compared with the value before administration. The H. pylori test, as well as the capsules containing $^{13}$C-urea and $^{13}$C-bicarbonate, were well tolerated by all volunteers.

**Availability of $^{13}$C (as $^{13}$C$_2$O$_2$) in blood**

The appearance of $^{13}$C-urea in blood is shown in Figure 2. It can be seen that the coated capsule was able to deliver the tracer in more distal segments of the GIT compared with the uncoated capsule. This is expressed in Table 2 by the $t_{\text{max}}$ of both capsules (mean difference: 284 min, 95%CI: 203–364, $P = 0.002$) and the lag time of the coated capsule (mean: 224 min, CV: 11.5%). The CV of the $t_{\text{max}}$ was considerably smaller for the coated capsule than for the CV of the uncoated capsule. The pulse time was around 120 min for subjects 1 to 3. Subject 4 showed a markedly shorter pulse time of 23 min. The UDV was on average 0.64 L·kg$^{-1}$ when $^{13}$C-urea was administered in coated capsules (mean difference: 1.5 h, 95%CI: 0.9–2.1, $P = 0.005$). The availability of $^{13}$C-urea in the UDV from coated capsules showed a range
Table 1 Quality control data for the uncoated and coated capsules

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Specification</th>
<th>Uncoated capsules</th>
<th>Coated capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Visual inspection</td>
<td>Smooth/not damaged</td>
<td>Complies</td>
<td>Complies</td>
</tr>
<tr>
<td>Uniformity of weight</td>
<td>Ph.Eur. 2.9.5</td>
<td>not more than 2 deviate</td>
<td>Complies</td>
<td>Complies</td>
</tr>
<tr>
<td>Uniformity of weight</td>
<td>LNA</td>
<td>CV ≤ 4.0%</td>
<td>Complies</td>
<td>Complies</td>
</tr>
<tr>
<td>Amount of coating</td>
<td>Weight</td>
<td>50–60 mg</td>
<td>NA</td>
<td>50 mg</td>
</tr>
<tr>
<td>Dissolution profile (16)</td>
<td>t_{5%} released</td>
<td>&gt;180 min</td>
<td>NA</td>
<td>210 min</td>
</tr>
<tr>
<td></td>
<td>Pulse time (t_{70%}−t_{50%})</td>
<td>&lt;60 min</td>
<td>NA</td>
<td>58 min</td>
</tr>
<tr>
<td></td>
<td>Cumulative release, t</td>
<td>&gt;80%</td>
<td>NA</td>
<td>97.9%</td>
</tr>
<tr>
<td>Disintegration time</td>
<td>Ph.Eur. 2.9.1</td>
<td>&lt;15 min</td>
<td>Complies</td>
<td>NA</td>
</tr>
</tbody>
</table>

LNA, Laboratorium Nederlandse Apothekers/Laboratory of Dutch Pharmacists; NA, not applicable.

Figure 2 Concentration in plasma of $^{13}$C-urea after intake of an uncoated capsule or a coated capsule. The concentration time curves are presented for each subject. Capsule with $^{13}$C-urea: coated (■) or uncoated (○).

Table 2 Release kinetic parameters derived from the $^{13}$C (as $^{13}$C-urea) measurements in plasma after intake of coated and uncoated capsules containing $^{13}$C-urea

<table>
<thead>
<tr>
<th>Subject</th>
<th>$t_{\text{max}}$ (min)</th>
<th>Half-life (h)</th>
<th>UDV (L·kg$^{-1}$)</th>
<th>$t_{\text{max}}$ (min)</th>
<th>Log time (min)</th>
<th>Pulse (min)</th>
<th>Half-life (h)</th>
<th>$F_{\text{not fermented}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>5.6</td>
<td>0.65</td>
<td>375</td>
<td>255</td>
<td>120</td>
<td>7.5</td>
<td>0.30</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>7.5</td>
<td>0.65</td>
<td>315</td>
<td>195</td>
<td>120</td>
<td>8.5</td>
<td>0.04</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>5.6</td>
<td>0.53</td>
<td>345</td>
<td>214</td>
<td>131</td>
<td>7.0</td>
<td>0.29</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>7.9</td>
<td>0.72</td>
<td>255</td>
<td>232</td>
<td>23</td>
<td>9.6</td>
<td>0.68</td>
</tr>
<tr>
<td>Average</td>
<td>39</td>
<td>6.6</td>
<td>0.64</td>
<td>323</td>
<td>224</td>
<td>98</td>
<td>8.1</td>
<td>0.33</td>
</tr>
<tr>
<td>CV (%)</td>
<td>46.1</td>
<td>18.5</td>
<td>12.4</td>
<td>15.9</td>
<td>11.5</td>
<td>51.7</td>
<td>13.8</td>
<td>80.6</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; UDV, urea distribution volume.

of 4–68% (average: 33%, CV: 80.6%). These values indicate that 4–68% of the administered 13C-urea had not been fermented.

### Availability of 13C (as 13CO2) in breath

Figure 3 shows the results from the breath measurements after intake of coated capsules containing 13C-bicarbonate or 13C-urea. Both substances, compounded in the capsules, appeared in breath after more than 3 h (Table 3, lag time). The observed difference between the lag times was not statistically significant (mean difference: 66 min, 95%CI: 0–32, \( P = 0.225 \)). Even when the lag time of subject 1 was not included in the statistical calculation, the lag times still were the same (mean difference: 16 min, 95%CI: 0–32, \( P = 0.051 \)). In all subjects, the availability of 13C (\( F_{\text{fermented}} \)) showed an average of 37.3% (CV: 40.3) when 13C-urea was administered in a coated capsule. This was less than the availability of 13C when administered as 13C-bicarbonate (mean: 55.0%). The difference in availability of 13C was about 17% and statistically significant (mean difference: 17.7, 95%CI: 0.1–35.3, \( P = 0.049 \)).

The pulse from coated capsules was faster for 13C-bicarbonate as compared with capsules containing 13C-urea (mean difference: 59.7 min, 95%CI: 20.4–99.1 min, \( P = 0.017 \)).

### Table 3

#### Release kinetic parameters derived from the 13C (as 13C-urea) measurements in breath after intake of coated capsules containing 13C-urea or 13C-bicarbonate

<table>
<thead>
<tr>
<th></th>
<th>Coated bicarbonate capsule</th>
<th>Coated urea capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>Cumulative PDR( t = 12 )</td>
<td>Pulse time (min)</td>
</tr>
<tr>
<td>Subject 1</td>
<td>83</td>
<td>55.6</td>
</tr>
<tr>
<td>Subject 2</td>
<td>213</td>
<td>55.6</td>
</tr>
<tr>
<td>Subject 3</td>
<td>186</td>
<td>65.9</td>
</tr>
<tr>
<td>Subject 4</td>
<td>199</td>
<td>42.7</td>
</tr>
<tr>
<td>Average</td>
<td>170</td>
<td>55.0</td>
</tr>
<tr>
<td>CV (%)</td>
<td>34.8</td>
<td>17.3</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; PDR, percentage dose recovered.
Validity of the model

All $^{13}$C administered was eventually recovered as is shown by the sum of the $F_{\text{fermented}}$ and $F_{\text{not fermented}}$. $F_{\text{total}}$ averages 99% (CV: 9.1%). Furthermore, the model showed (Figure 4) a very high inverse correlation between $F_{\text{not fermented}}$ and $F_{\text{fermented}}$ (corrected, respectively, not corrected for CO$_2$ retention) as expressed by Pearson’s $r$ values of $-0.981$ ($P = 0.06$) or $-0.942$ ($P = 0.02$).

Discussion and conclusions

The present study shows the applicability of $^{13}$C-urea as a marker substance for the assessment of in vivo behaviour of oral colon-targeted dosage forms. We combined conventional kinetic assessment by plasma concentration versus time curves with a stable isotope technology indicating the segment where release occurs. $^{13}$C-urea served both as model substance for kinetic assessment as well as the stable isotopic marker. $^{13}$C-urea fulfills both roles based on the combination of suitable physico-chemical, kinetic characteristics and excellent safety profile. First, urea is freely soluble in water (1 g·mL$^{-1}$). Based on the Rule of 5 (Lipinski et al., 1997), $^{13}$C-urea is classified as a class I substance in the Biopharmaceutic Classification System (BCS) (Amidon et al., 1995). Therefore, $^{13}$C-urea is expected to permeate rapidly through the intestinal wall into the blood circulation. This expectation is confirmed by the absorption of $^{13}$C-urea from the uncoated capsules (Table 2: uncoated capsules). According to the BCS, the rate-limiting step for absorption of class I substances is the gastric transit time when it is administered in an uncoated capsule or, accordingly, the GI transit time when it is administered in a coated capsule. No degradation occurred in the upper GIT nor en route to the blood circulation. Second, its distribution was favourable. The volume of distribution was $-0.6$ L·kg$^{-1}$, indicating uniform distribution over the water compartment in the human body. This validates our choice to perform calculations by a one-compartment model. Third, $^{13}$C-urea may be absorbed from the GIT, unchanged or after fermentation, depending on the presence of urease and its activity. As urea is the end product of nitrogen physiology, no relevant amount of metabolite is formed after absorption of this compound in its unchanged form. This simplifies the interpretation of the experimental results. Proof of this was found in the breath test data of the uncoated capsules, which did not reveal any significant excess of $^{13}$C in breath (data not shown). As can be observed from the breath test data for the coated capsules, in the distal parts of the intestine, $^{13}$C-urea was fermented by urease activity. $^{13}$C was absorbed from the intestinal lumen as $^{13}$C-bicarbonate and subsequently exhaled as $^{13}$CO$_2$. Fourth, urea is eliminated mainly by renal excretion. This enables easy (non-invasive) sampling and thus, quantification of the elimination routes. Fifth, urea exerts no pharmacological effects, which makes it a very attractive marker for clinical trials especially with children and healthy volunteers.

We checked the internal validity of the kinetic model by calculating the mass balance of administered $^{13}$C-urea. The sum of the $F_{\text{fermented}}$ (corrected for CO$_2$ retention) and $F_{\text{not fermented}}$ was on average 99%. This shows that the model takes into account all significant kinetic routes of $^{13}$C-urea. Furthermore, the data relate to each other in the right order (‘temporal precedence’). In all four subjects, the pulse of $^{13}$C-urea compared with $^{13}$C-bicarbonate (both from coated capsules) was slower (as expressed by a higher pulse time). This may be explained by the fact that urea needs to be fermented by urease before it becomes available in the lumen as $^{13}$C-bicarbonate. This additional conversion requires, of course, some extra time due to uptake of urea by the bacteria, enzymatic conversion, excretion of CO$_2$ and equilibration of CO$_2$ with bicarbonate. Based on the quality control data, it may be hypothesized that the slower release of $^{13}$C-urea from the coated capsule may explain the slower pulse (Table 1). However, disintegration of the coating and first release is expected to start already in the terminal ileum (Schellekens et al., 2008), which is a urease-poor segment. In general, the kinetics of $^{13}$CO$_2$ appearance in breath is determined by a combination of factors involving release of $^{13}$C-urea, fermentation by bacterial urease as well as secretion, equilibration and transport of $^{13}$CO$_2$. A difference in dissolution profile is not a probable major explanation for the slower pulse in the urease-rich segments as shown by our $^{13}$CO$_2$ results.

Furthermore, the $F_{\text{fermented}}$ (not corrected for CO$_2$ retention) for $^{13}$C-urea in coated capsules was lower than for $^{13}$C-bicarbonate in coated capsules. This may be the result of some extra time due to uptake of urea by the bacteria, enzymatic conversion, excretion of CO$_2$ and equilibration of CO$_2$ with bicarbonate. Based on the quality control data, it may be hypothesized that the slower release of $^{13}$C-urea from the coated capsule may explain the slower pulse (Table 1). However, disintegration of the coating and first release is expected to start already in the terminal ileum (Schellekens et al., 2008), which is a urease-poor segment. In general, the kinetics of $^{13}$CO$_2$ appearance in breath is determined by a combination of factors involving release of $^{13}$C-urea, fermentation by bacterial urease as well as secretion, equilibration and transport of $^{13}$CO$_2$. A difference in dissolution profile is not a probable major explanation for the slower pulse in the urease-rich segments as shown by our $^{13}$CO$_2$ results.

The lag times, as determined for the appearance of $^{13}$C in breath or plasma after intake of a coated capsule containing $^{13}$C-urea, showed little difference. This points to the fact that fermentation and absorption occur simultaneously. Because
urea may be absorbed both from the small intestine and from the caecum and colon (Billich and Levitan, 1969; Moran and Jackson, 1990), no concluding statements can be made with respect to the location of the absorption process of intact urea. However, when the $^{13}$C-recoveries from blood and breath after intake of a coated capsule with $^{13}$C-urea are analysed head to head and in more detail (Figures 2 and 3), it is observed that $^{13}$C-urea appeared in the circulation around the moment the subsequent meal is taken. Then, shortly after the appearance of $^{13}$C-urea in blood and after intake of the subsequent meal, $^{13}$C was also detected in breath. This observation leads to the hypothesis that nearly all $^{13}$C-urea had been released in the terminal ileum and that some absorption takes place at that site. Then, the intake of the subsequent meal induced the transfer of the remaining $^{13}$C-urea into the urease-rich segments (caecum or colon). Absorption of intact $^{13}$C-urea continues, but now, bacteria start to ferment $^{13}$C-bicarbonate, which is subsequently absorbed as $^{13}$C-bicarbonate.

No clear relationship could be observed between the lag times and the $F_{\text{terminal}}$. The lack of relationship can be understood as a consequence of the experimental setup; the lag times are controlled (i.e. maximized at 3 h) by taking the second meal (Priebe et al., 2004; 2006; Schellekens et al., 2008).

Moreover, the trend line (Figure 4) depicts the inverse relation between the availabilities of fermented and not fermented urea. It shows that when no urea is fermented, all is absorbed. In addition, when 100% of the urea is delivered in urease-rich segments, little resorption of not fermented $^{13}$C-urea is to be expected. All four cases fit well into the model, as is concluded from the Pearson’s $r$ values. This opens the possibility to evaluate colon delivery dosage forms by collecting breath samples only. Urine samples instead of blood samples may be used to verify that all urea is released.

The external validity of the model determines whether the kinetic model may be used to investigate both the release kinetics of coated dosage forms and the GI segment of release.

First, the kinetic profile of coated dosage forms may be monitored based on only breath samples. The lag time and $F_{\text{terminal}}$ (and its corresponding $F_{\text{not terminal}}$) can be obtained. In our experimental setup, a subsequent meal was used in order to standardize and control the intestinal transit of the capsule. As this standardized subsequent meal is not present in clinical practice, it should preferably be omitted when carrying out a bioavailability study. Second, any statement on $^{13}$C-urea absorption and fermentation on these assessments. Evaluation of colon-targeted oral dosage forms may be performed by analysing the $^{13}$C-appearence in breath only. This opens new possibilities in performing biopharmaceutical studies to improve the therapy of diseases occurring locally in the colon or in which drugs are used that require colonic delivery.

Acknowledgement

We thank Dr V Fidler for his valuable comments on the statistical calculations.

Conflict of interest

None.

References


