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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 ($F_{fermented}$) and the appearance of ^{13}C in blood as ^{13}C-urea ($F_{not\ fermented}$) show a high inverse correlation (Pearson's $r = -0.981$, $P = 0.06$). The total recovery of ^{13}C ($F_{fermented} + F_{not\ fermented}$) 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.
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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, γ-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,

Correspondence: RCA Schellekens, Department of Clinical and Hospital Pharmacy, University Medical Center Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands. E-mail: r.c.a.schellekens@apoth.umcg.nl
*Both authors share first authorship
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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)

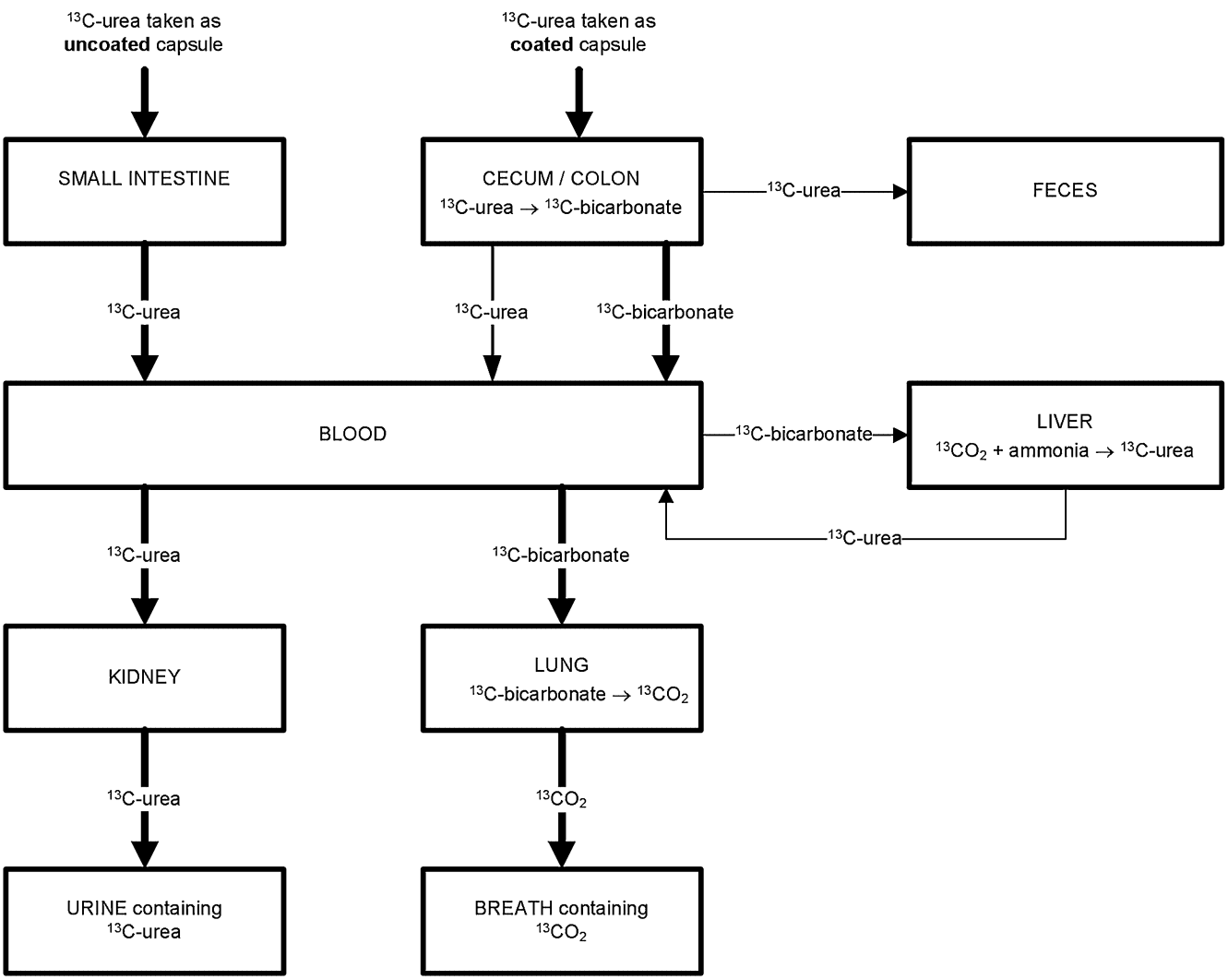

Figure 1 Absorption, metabolism and elimination of ^{13}C-urea upon release in the small intestine versus release in the caecum or colon. The weight of the line symbolizes the relative importance of the corresponding kinetic process. ^{13}C-urea delivered in the small intestine (urease-poor segment) will be absorbed intact instead of being fermented. ^{13}C-urea available in the caecum or colon (urease-rich segment) will be converted into $^{13}CO_2$/^{13}C-bicarbonate by *in situ* fermentation by the bacterial ureases. This molecule will subsequently be absorbed into the blood circulation as ^{13}C-bicarbonate. ^{13}C-bicarbonate may also be made available in the caecum or colon directly from a coated capsule. The ^{13}C-bicarbonate will follow the same kinetic route as *in situ* formed ^{13}C-bicarbonate.

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 appendectomia). 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 orocaecal 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-lithium 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 collected before administration and at regular time intervals after capsule administration up to 14 h (^{13}C-urea capsule) or 9 h (^{13}C-bicarbonate capsule). After intake of the coated urea capsule, one sample of blood and breath was taken the next morning. In the case of ^{13}C-urea containing capsules, blood samples were collected at regular intervals up to 12 h after intake. $^{13}C/^{12}C$ isotope ratios (expressed as $\delta^{13}C_{PDB}$ values in per mil difference vs. the Pee Dee Belemnite standard) in the CO_2 of the breath samples were analysed by using a validated breath ^{13}C-analyser (Heliview, Medichems, Seoul, South Korea) based on isotope ratio mass spectrometry (IRMS) (Stellaard and Geypens, 1998). Blood samples were centrifuged and plasma was stored at –20°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 $\delta^{13}C_{PDB}$ 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):

$$(^{13}C-urea)_t = \frac{APE\ ^{13}C_t \times (urea)_t}{100} \quad (1)$$

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

$$\log(^{13}C-urea)_t = \log(^{13}C-urea)_{t=0} - a \times t \quad (2)$$

where a is the slope of the curve.

Subsequently, several kinetic parameters were deducted, 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 ($t_{5\%}$). Based on Equation (2), the intercept with the y-axis (t = 0 h) of the curve in the steady state phase represents the fictitious $(^{13}C\text{-urea})_{t=0}$ 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):

$$UDV = \frac{Dose(^{13}C-urea)}{(^{13}C-urea)_{t=0} \times LBM_{subject}} \quad (3)$$

Then, the elimination rate constant (k_{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.

$$k_{el} = 2.303 \times a \quad (4)$$

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

$$t_{1/2} = \frac{0.693}{k_{el}} \quad (5)$$

The pulsatile release properties are reflected by the so-called pulse time, defined as the time period between the lag time ($t_{5\%}$) and t_{max}. The availability of ^{13}C-urea in urease-poor segments is expressed by the not fermented fraction ($F_{not\ fermented}$).

The $F_{\text{not fermented}}$ is calculated relative to the bioavailability of ^{13}C-urea administered in an uncoated capsule according to Equation (6):

$$F_{\text{not fermented}} = \frac{\sum_{t=lag\ time}^{t=lag\ time+5\,\text{h}} AUC_{\text{coated}}}{\sum_{t=0\,\text{h}}^{t=5\,\text{h}} AUC_{\text{uncoated}}} \times F_{\text{uncoated}} \quad (6)$$

The AUC of ^{13}C-urea from the coated capsule 5 h after the lag time is related to the AUC of ^{13}C-urea from the uncoated capsule 5 h after the time point of administration. ^{13}C-urea from the uncoated capsule is released in the proximal segments of the GIT (stomach, duodenum) and absorbed into the circulation by passive transport over the intestinal wall. The bioavailability (F_{uncoated}) 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 $\delta^{13}C_{PDB}$ 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 $\text{cumPDR}_{t\,=\,12\,\text{h}}$ 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 $\text{cumPDR}_{t\,=\,12\,\text{h}}$ obtained applying ^{13}C-bicarbonate as the tracer compound is considered to be the maximal obtainable recovery in breath of $^{13}CO_2$ 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 $t_{\max}$ and $t_{5\%}$.

$$F_{\text{fermented}} = \frac{cumPDR_{t=12\,\text{h}}(^{13}C-urea)}{cumPDR_{t=12\,\text{h}}(^{13}C-bicarbonate)} \times 100\% \quad (7)$$

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_{total} was close to 100%.

Statistical procedures

The results were analysed by descriptive statistics. Furthermore, we used the paired *t*-test to compare the behaviour of the two types of capsules (two-tailed, α = 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*).

Materials

Polyethyleneglycol 6000, acetone, colloidal anhydrous silica (BUFA, IJsselstein, The Netherlands), microcrystalline cellulose (Avicel PH102, FMC Biopolymer, Philadelphia, PA, USA), croscarmellose sodium (Ac-di-sol, FMC Biopolymer) and methacrylic acid-methyl methacrylate copolymer 1:2 (Eudragit S100, Röhm, Darmstadt, Germany) were obtained via a certified wholesaler (Spruyt-Hillen, IJsselstein, The Netherlands). Hard gelatine capsules were obtained from Lamepro (Raamsdonksveer, The Netherlands). Water for injections was obtained from Fresenius Kabi (Bad Homburg, Germany). All ingredients were of pharmacopoeial grade (Ph.Eur.). The stable isotope labelled ^{13}C-urea (AP 99%) and sodium ^{13}C-bicarbonate (AP 99%) were obtained from an FDA-controlled facility (Isotec, Fort Myers, FL, USA). Hard gelatine capsules (size 2) containing the required marker ingredients (100 mg ^{13}C-urea or 100 mg ^{13}C-sodium bicarbonate) were prepared by normal compounding procedures. The capsules were manually filled with a premix of active ingredient and excipients. A coating was applied to get delivery of the tracers in the distal parts of the small intestine and the proximal parts of the colon. The composition of the coating used in this study was Eudragit S : PEG 6000 : Ac-di-sol = 58.3%:8.3%:33.3% w/w. The coating procedure has been described before (Schellekens *et al.*, 2008). Coating thickness was calculated and expressed as the amount of Eudragit S applied per cm^2. The uncoated and coated capsules met established quality control criteria (Table 1).

Results

All subjects were tested negative for the presence of *H. pylori*, meaning that the $\delta^{13}C_{PDB}$ abundance in breath CO_2 30 min after administration of ^{13}C-urea as an oral solution was less than 5‰ increased 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-urea) 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 $t_{\max}$ 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 $t_{\max}$ 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 $L \cdot kg^{-1}$ (CV: 12.4%). The elimination of ^{13}C-urea, as expressed by the half-life, was slower 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

Parameter	*Method*	*Specification*	*Uncoated capsules*		*Coated capsules*	
			Batch ^{13}C-urea	*Batch ^{13}C-bicarbonate*	*Batch ^{13}C-urea*	*Batch ^{13}C-bicarbonate*
Appearance	Visual inspection	Smooth/not damaged	Complies	Complies	Complies	Complies
Uniformity of weight	Ph.Eur. 2.9.5	not more than 2 deviate	Complies	Complies	Complies	Complies
Uniformity of weight	LNA	CV ≤ 4.0%	Complies	Complies	Complies	Complies
Amount of coating	Weight	50–60 mg	NA	NA	50 mg	59 mg
Dissolution profile (16)	$t_{5\%\ released}$	>180 min	NA	NA	210 min	220 min
	Pulse time ($t_{70\%}$–$t_{5\%}$)	<60 min	NA	NA	58 min	20 min
	Cumulative release$_{t\ =\ 360\ min}$	>80%	NA	NA	97.9%	101.6%
Disintegration time	Ph.Eur. 2.9.1	<15 min	Complies	Complies	NA	NA

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

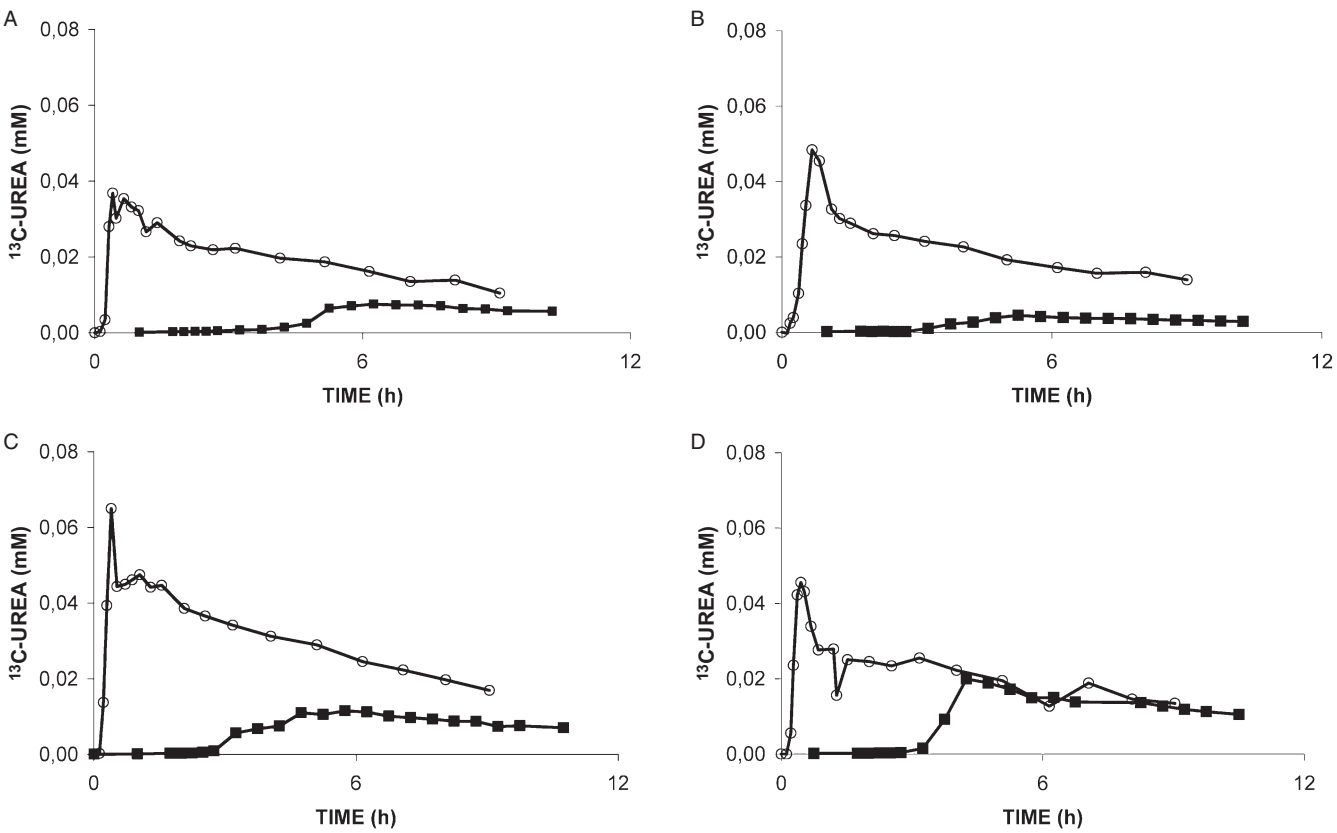

Figure 2 Concentration in plasma of ^{13}C-urea after intake of an uncoated capsule or a coated capsule. The concentration time curves are presented for each subject. Capsule with ^{13}C-urea: coated (■) or uncoated (○).

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

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

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

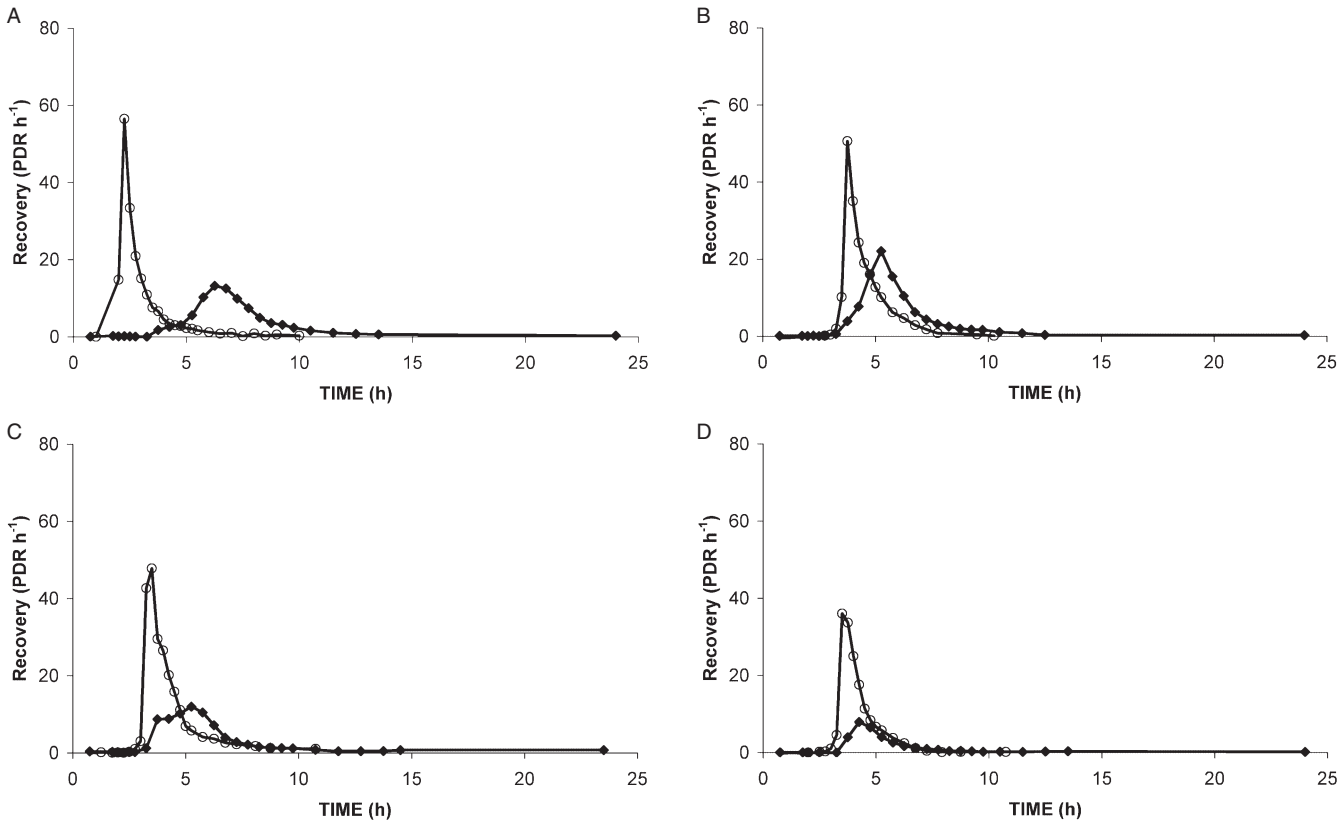

Figure 3 Recovery in breath of ^{13}C after intake of a coated capsule with ^{13}C-bicarbonate or ^{13}C-urea.The recovery time curves are presented for each subject. Capsule with ^{13}C-bicarbonate (○) or ^{13}C-urea (◆). PDR, percentage dose recovered.

Table 3 Release kinetic parameters derived from the ^{13}C (as ^{13}C-urea) measurements in breath after intake of coated capsules containing ^{13}C-urea or ^{13}C-bicarbonate

	Coated bicarbonate capsule			*Coated urea capsule*			
	Lag time (min)	*Cumulative PDR$_{t=12}$*	*Pulse time (min)*	*Lag time (min)*	*Cumulative PDR$_{t=12}$*	*Pulse time (min)*	*F$_{fermented}$*
Subject 1	83	55.6	52	262	43.0	113	0.77
Subject 2	213	55.6	12	236	51.1	79	0.92
Subject 3	186	65.9	24	206	38.8	109	0.59
Subject 4	199	42.7	11	218	16.1	37	0.38
Average	170	55.0	25	231	37.3	85	0.67
CV (%)	34.8	17.3	77.2	10.6	40.3	41.6	35.0

CV, coefficient of variation; PDR, percentage dose recovered.

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

Availability of ^{13}C (as $^{13}CO_2$) in breath

Figure 3 shows the results from the breath measurements after intake of coated capsules containing ^{13}C-bicarbonate or ^{13}C-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: 60 min, 95%CI: –66 to 186, $P = 0.225$). 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 ^{13}C ($F_{fermented}$ but not corrected for CO_2 retention) showed an average of 37.3% (CV: 40.3) when ^{13}C-urea was administered in a coated capsule. This was less than the availability of ^{13}C when administered as ^{13}C-bicarbonate in a coated capsule (mean: 55.0%). The difference in availability of ^{13}C was about 17% and statistically significant (mean difference: 17.7, 95%CI: 0.1–35.3, $P = 0.049$). The availability of ^{13}C-urea ($F_{fermented}$ corrected for CO_2 retention) had an average of 67% (CV: 35.0%). The pulse from coated capsules was faster for ^{13}C-bicarbonate as compared with capsules containing ^{13}C-urea (mean difference: 59.7 min, 95%CI: 20.4–99.1 min, $P = 0.017$).

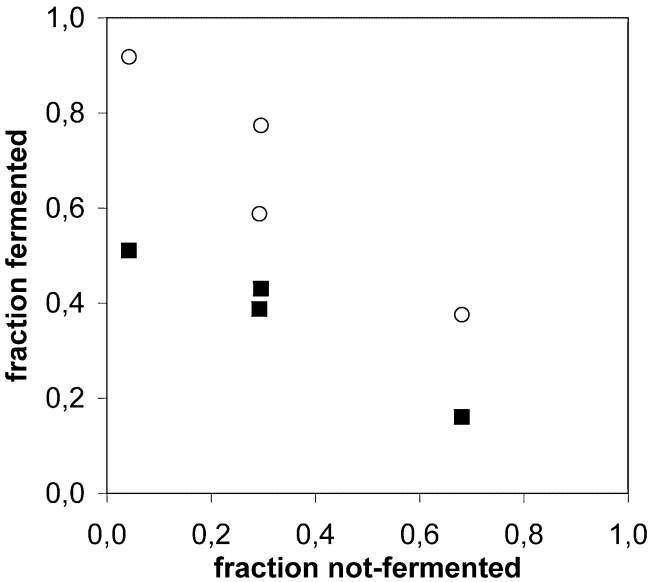

Figure 4 Relationship between $F_{\text{not fermented}}$ and $F_{\text{fermented}}$. Fraction fermented: (○) corrected for CO_2 retention and (■) not corrected for CO_2 retention.

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_{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 various factors. A (small) part of the liberated urea escapes fermentation by being absorbed quickly or by being utilized as a substrate by bacteria to form other metabolites than CO_2 and NH_3. Urea is also being lost through the faeces in a small amount (<4%) (Billich and Levitan, 1969; Moran and Jackson, 1990).

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-urea into $^{13}CO_2$, which is subsequently absorbed as ^{13}C-bicarbonate.

No clear relationship could be observed between the lag times and the $F_{fermented}$. 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 $F_{fermented}$ (and its corresponding $F_{not\ fermented}$) 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 the availability in any GI segment depends on the biological dogma that fermentation does not occur in the small intestine as it is a urease-poor segment. A wealth of information is available on fermentation processes in the GIT (Cummings *et al.*, 2001; Wong and Jenkins, 2007), and all studies conclude that under physiological situations (i.e. no bacterial overgrowth), no relevant fermentation occurs in the small intestine. Furthermore, urease is not produced by humans but only by bacteria, yeasts and several higher plants. Therefore, ^{13}C-urea is considered a safe marker substance, able to provide information on the GI segment of release in humans.

In conclusion, we performed the first proof-of-concept study on the application of ^{13}C-urea as a marker substance for effective colon delivery. It was shown that ^{13}C-urea is able to provide information on both the release kinetics of a colon-targeted oral dosage form and the GI segment of release. ^{13}C-urea fulfils both roles based on the combination of suitable physico-chemical and kinetic characteristics as well as an excellent safety profile. Further validation studies in healthy volunteers and patients should evaluate its potential in assessing the performance of colon delivery dosage forms and the impact of inter-individual and intra-individual variance in ^{13}C-urea absorption and fermentation on these assessments. Evaluation of colon-targeted oral dosage forms may be performed by analysing the ^{13}C-appearance 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.

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Conflict of interest

None.

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