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RESEARCH PAPER

A Non-Invasive, Low-Cost Study Design to Determine the Release Profile of Colon Drug Delivery Systems: A Feasibility Study

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ABSTRACT

Purpose Conventional bioavailability testing of dosage forms based on plasma concentration-time graphs of two products in a two-period, crossover-design, is not applicable to topical treatment of intestinal segments. We introduce an isotope dual-label approach (^{13}C- and $^{15}N_2$-urea) for colon drug delivery systems that can be performed in a one-day, non-invasive study-design.

Methods Four healthy volunteers took an uncoated or a ColoPulse-capsule containing ^{13}C-urea and an uncoated capsule containing $^{15}N_2$-urea. In case of colon-release ^{13}C-urea is fermented and ^{13}C detected as breath $^{13}CO_2$. Absorbed ^{13}C-urea and ^{15}N-urea are detected in urine.

Results C and ^{15}N in urine released from uncoated capsules showed a ratio of 1.01 ± 0.06. The $^{13}C/^{15}N$-recovery ratio after intake of a ColoPulse-capsule was constant and lower >12 h post-dose (median 0.22, range 0.13–0.48). The $^{13}C/^{15}N$-ratio in a single urine sample at $t \geq 12$ h predicted the 24 h non-fermented fraction ^{13}C of <26 %. Breath $^{13}CO_2$ indicated delayed (>3 h) release and a fermented fraction ^{13}C >54 %.

Conclusions Breath and urine ^{13}C and ^{15}N data describe the release-profile and local bioavailability of a colon delivery device. This allows non-invasive bioavailability studies for evaluation of colon-specific drug delivery systems without radioactive exposure and with increased power and strongly reduced costs.

KEY WORDS colon-delivery · local bioavailability · release profile · stable isotope · urea

ABBREVIATIONS

AUC	area under the curve
BA	bioavailability
BE	bioequivalence
C_{max}	maximal concentration
IRMS	isotope ratio mass spectrometry
PDR	percentage of dosage recovered

INTRODUCTION

Investigation of the bioavailability is an early step in the clinical development of a new drug product or drug delivery system. The United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have released guidelines for bioavailability testing of drug products which aim for systemic exposure of the drug substance (1,2). The conventional systemic bioavailability study design is a two-sequence, two-period crossover design, where blood pharmacokinetic parameters as the maximal concentration

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(C_{max}) and the area under the concentration–time curve (AUC) play a pivotal role. This conventional approach however is not applicable to drug delivery systems which aim for delivery of the drug substance in a specific intestinal segment for topical treatment. Examples are 5-aminosalicylic acid or immunosuppressant formulations such as budesonide for the treatment of inflammatory bowel disease. In these specific cases it is not relevant to investigate systemic bioavailability. To the best of our knowledge no international guidelines are available describing a consensus approach to evaluate local bioavailability. In the international literature a myriad of approaches are described to determine intestinal drug delivery. Pharmacokinetics is often combined with imaging technologies to localize release, such as endoscopy, radiology, gamma scintigraphy (3–5) or MRI (6,7). Stable isotope technology is also mentioned in this context (8).

In two earlier studies we determined the local bioavailability and release profile of a coated capsule which acts as colon-specific drug delivery system (the ColoPulse-system) using stable isotope technology (9,10). The ColoPulse system is characterized by a pulsatile release of its contents at pH>7.0. The coating consists of a mixture of Eudragit S-100:PEG 6000:Ac-di-sol (58.3 %:8.3 %:33.3 % w/w) (11). The first paper describes a proof-of-concept study in which it was shown that ^{13}C-urea was able to provide information on both the release kinetics of a ColoPulse-capsule and the gastro-intestinal segment of release. The second paper describes a single dose two-period crossover study in which ^{13}C-urea was used as the marker substance. In this study an uncoated capsule was taken on day 1 (as a reference) and a ColoPulse-capsule on day 8. The delivery in the colon by the ColoPulse-capsule was monitored by measuring the $^{13}CO_2$ response in breath produced by bacterial fermentation in the colon of ^{13}C-urea. Local bioavailability was determined by recovery of ^{13}C in breath. Total recovery was quantified by the sum of recoveries of ^{13}C in breath and blood or urine. A strong correlation (r=0.943) was found between blood and urine kinetics, indicating that non-invasive urine sampling could replace blood sampling.

We hypothesized that investigation of local bioavailability and determination of the release profile of ColoPulse-capsules could be improved by application of a dual-label isotope strategy. This approach permits a one-day study design and non-invasive sampling. A ColoPulse-capsule containing ^{13}C-urea and an uncoated capsule containing $^{15}N_2$-urea (as a reference) are taken simultaneously. Release of ^{13}C- or $^{15}N_2$-urea in the small intestine (urease-poor region) from an uncoated capsule leads to the recovery of unaltered ^{13}C- or $^{15}N_2$-urea in urine. Release of ^{13}C-urea in the ileocolonic intestinal segment (urease-rich region) from a ColoPulse-capsule leads to *in situ* fermentation of ^{13}C-urea into $^{13}CO_2$ followed by exhalation of in breath. Local bioavailability in the colon can be described by the difference between kinetics of $^{15}N_2$- and ^{13}C-urea (Fig. 1). The differential kinetics of these isotopically labeled substances can potentially describe both release kinetics and the gastrointestinal segment of release. Using this strategy, the clinical trial can be shortened to a one-period design and the sample load can be reduced by 50 %. As a consequence the cost of a bioavailability trial is reduced. In addition, the influence of day-to-day variation in urea kinetics is eliminated, which increases the power of the study. Furthermore less subjects need to be included, which further reduces the cost of the clinical trial.

In this paper we describe a proof-of-concept study to demonstrate the feasibility of the dual-isotope strategy to determine the release profile of ColoPulse-capsules in a one-day, non-invasive study design.

MATERIALS AND METHODS

Chemicals, Drug Substances, and Drug Products

Polyethylene glycol 6000, acetone, colloidal anhydrous silica (BUFA, The Netherlands), microcrystalline cellulose (Avicel PH102, FMC Biopolymer, USA), croscarmellose sodium (Ac-di-sol, FMC Biopolymer, USA), methacrylic acid-methyl methacrylate copolymer 1:2 (Eudragit S100, Röhm, Germany), were obtained via a certified wholesaler (Spruyt-Hillen, The Netherlands). Hard gelatine capsules (size 2) were obtained from Lamepro (The Netherlands). Water for injections was obtained from Fresenius Kabi (Germany). All ingredients were of pharmacopoeial grade (Ph. Eur.). The stable Isotope labelled ^{13}C-urea and ^{15}N-urea (AP 99 %) was obtained from an FDA-controlled facility (Isotec, USA). Hard gelatine capsules containing 100 mg ^{13}C- or 50 mg $^{15}N_2$-urea were prepared according to the compounding procedures of the Laboratory of Dutch Pharmacists (LNA). The capsules were manually filled with a premix of ^{13}C-urea or $^{15}N_2$-urea and excipients. A coating was applied using the ColoPulse technology (11). Coating thickness was calculated and expressed as the amount of Eudragit S-100 applied per cm^2. The coated capsules met established quality control criteria (Table I). The pulsatile release properties are reflected by the so-called pulse-time, defined as the period between the lag time (=$t_{5\ \%\ release}$) and $t_{70\ \%}$ release.

Subjects

Four healthy volunteers (one female, three males, age 30, 39, 50, 61 years) participated in the study. They had neither history of gastrointestinal diseases (ulcerative colitis, Crohn's disease, spastic colon, colon cancer, ileus, stoma, stomach- and/or intestinal infection) nor of gastrointestinal surgery.

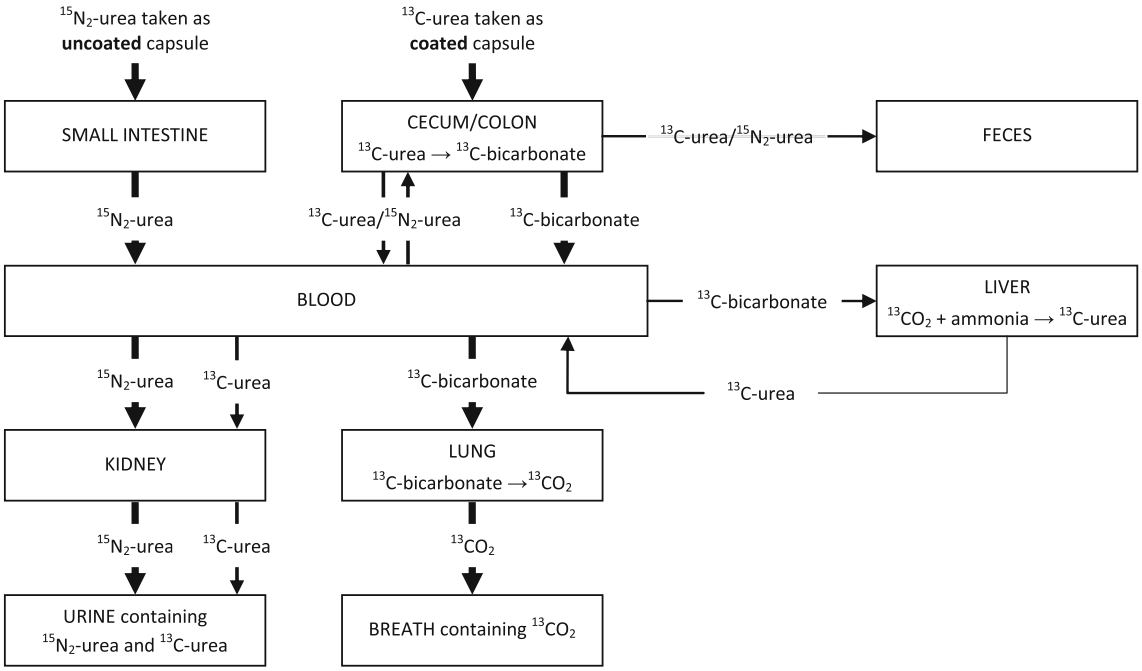

Fig. 1 Absorption, metabolism and elimination of ^{13}C-urea and ^{15}N2-urea. The weight of the *arrow* symbolizes the importance of the kinetic process.

They did not use antibiotics or drugs influencing the gastrointestinal transit time for at least 3 months before start of the study. A possible *Helicobacter pylori* infection was excluded. The study design was approved by the ethical committee of the University Medical Center Groningen and the study was performed according to principles of the Declaration of Helsinki.

Study Design

The clinical study consisted of two experiments. In the first experiment two uncoated capsules containing 50 mg $^{15}N_2$-urea and 100 mg ^{13}C-urea respectively were taken simultaneously in order to compare the kinetic behaviour of ^{13}C-urea and $^{15}N_2$-urea affected by absorption, distribution, metabolism and elimination. In the second experiment an uncoated capsule containing 50 mg $^{15}N_2$-urea and a ColoPulse-capsule containing 100 mg ^{13}C-urea were taken simultaneously. The second experiment aimed to give information on the release of ^{13}C-urea in the ileocolonic intestinal segment (urease-rich region) and of $^{15}N_2$-urea in the proximal small intestine (urease poor region) respectively. During the experiments the subjects feeding and drinking were standardized as described before (8,9). The subjects were fasted on day 1 from 20:00 h. Only water and tea without sugar were allowed. In the morning on day 2, they received the capsules together with 200 ml apple juice. After a period of 3 h a standardized breakfast consisting of a double sandwich was consumed in order to control the oro-cecal transit time. 5 and 10 h after intake of the capsules lunch and dinner were allowed. There were no food-restrictions except foods enriched in ^{13}C like corn, cane sugar and pineapple. During the day only water and tea without sugar were allowed. The study ended at 8.00 h on day 3.

Table I Quality Control Data of the Capsules

Parameter	Specification	Result
Variation of mass (capsules, uncoated, n=20)	<4 %	1.52 %
Variation of mass (capsules, coated, n=20)	<4 %	1.59 %
Coat thickness (mg Eudragit S/cm^2) (n=20)	Not applicable	9.8
Bursts or cracks in coating (n=6)	None	None
Lag time (minutes) (n=6)	>180	220
Pulse time (minutes) (n=6)	<60	38
Release at t_{360min} (n=6)	>80 %	107.9 %

Sample Collections and Analysis

Breath samples were collected every 30 min from 30 min before up to 15 h after intake of the capsules and were analysed as described before (8,9). Briefly, $^{13}C/^{12}C$ isotope ratios in the CO_2 of breath samples were analysed by using a validated breath ^{13}C-analyser (Thermo Fisher Scientific, Bremen, Germany) based on isotope ratio mass spectrometry (IRMS). Urine samples were collected during 24 h at prescribed intervals (0–4, 4–8, 8–12, 12–16 and 16–24 h) in 200 ml containers each containing 650 µl 6 M HCl. Urine volumes were recorded and 20 ml samples were stored at

−20 °C until analysis. The remaining urine was pooled and a 20 ml sample was stored at −20 °C. The pooled urine volume was considered as a the 24 h collection and used as gold standard for modeling (Statistical Procedures and Modeling section).

Concentrations of total N and C were determined based on element analysis. Urine aliquots of 25 µl were combusted in an elemental analyzer SL™ (SerCon, Crewe, UK) using copper oxide at 900 °C to NO_x and CO_2. NO_x was subsequently reduced to nitrogen gas over copper at 600 °C. Thereafter the ^{13}C and ^{15}N enrichments were measured online by IRMS (Tracer mass 20–20™, SerCon, Crewe, UK).

Data were expressed either as enrichment, as atom percent excess (APE) or as percentage of the dose ^{15}N or ^{13}C recovered (PDR). The method of urine sample preparation and IRMS-analysis was tested for accuracy (recovery), precision and linearity. This test was performed by spiking equal volumes (100 ml) of urine collected by one subject during 24 h with fixed amounts of $^{15}N_2$-urea (5 mg) and increasing amounts of ^{13}C-urea (0–10 mg).The theoretical ratios of $^{13}C/^{15}N$ in these samples were 0, 0.1, 0.2, 0.4, 0.6, 0.8,1.0.

Calculations

The percentage of the dose recovered (PDR) of ^{13}C and ^{15}N in each urine sample, the ratio of the PDRs from ^{13}C *versus* ^{15}N-ratio (the $^{13}C/^{15}N$-ratio), the fermented ($F_{fermented}$) and non-fermented ($F_{non\text{-}fermented}$) fraction of ^{13}C- urea were calculated as described before (10). In short, the fermented fraction was calculated as the cumulative PDR as ^{13}C in breath over the 15 h time period. The non-fermented fraction was calculated as the ratio of the percentage of the dose recovered as ^{13}C and ^{15}N (ratio $^{13}C/^{15}N$) in the 24 h urine collection. Total recovery was expressed as $F_{non\text{-}fermented} + F_{fermented}$.

Statistical Procedures and Modeling

The results were evaluated by descriptive statistics. The center was characterized by the arithmetic mean and median. The dispersion was characterized by the coefficient of variation (CV) and range correspondingly. A Wilcoxon matched-pairs test (two tailed, α=0.05) was used to compare the ratio $^{13}C/^{15}N$-ratio in the 24 h urine collection with the calculated ratio from a single urine sample and 95 % confidence intervals were established.

The correlation between the $^{13}C/^{15}N$-ratio in the 24 h urine collection and in a single urine collection collected at a time point ≥12 h post dose was investigated. The algorithm was obtained from the regression line. The correlation coefficient was calculated from the determination coefficient of the regression line.

Endpoints

The first endpoint of the study was to determine that ^{13}C-urea and $^{15}N_2$-urea exhibit the same kinetic properties in terms of absorption, distribution and elimination. The second was to show that a reduced PDR of ^{13}C-urea in urine is an indicator of bacterial fermentation of ^{13}C-urea delivered in the colon. The third endpoint was the total recovery of the ^{13}C-labeled atom to confirm that all elimination routes are covered by our sampling plan.

RESULTS

Urine Spiking Experiment

The method of sample preparation and IRMS analysis showed a recovery of 98±3.7 % for the ^{15}N- (n=7) and 94±2.2 % (n=6) for the ^{13}C-isotope. The precision was 3.8 % (n=7) for ^{15}N and 2.6 % (n=6) for ^{13}C. Furthermore, the method was linear in a range of 0 to 100 mg ^{13}C-urea/L (slope = 0.95, r^2=0.9987). The ratio $^{13}C/^{15}N$ was linear in a range of 0 to 1.0 (slope = 0.98, r^2=0.9999) when the measured ratio was plotted against the theoretical value.

In Vivo Experiment

Urine Data

In Fig. 2 the ratio of the PDRs of ^{13}C and ^{15}N measured in the urine samples is shown as a function of time. The $^{13}C/^{15}N$-ratio from uncoated capsules showed a mean ratio of 1.01±0.06 (n=20) during the first 24 h. The $^{13}C/^{15}N$-ratio after intake of the ColoPulse-capsule showed larger interindividual variation but remained constant in all subjects subject after 12 h post dose (median 0.22, range 0.13–0.48). The $^{13}C/^{15}N$-ratio in the 24 h urine collection after intake of the coated capsules (median 0.15, range 0.09–0.32) was lower than the ratio measured in the single urine samples after 12 h post dose, in all four cases.

The cumulative percentage non-fermented ^{13}C- and $^{15}N_2$-urea expressed as percentage of the dose recovered (PDR) per collected urine volume is shown for each subject in Fig. 3. The cumulative PDR of ^{13}C and ^{15}N in urine after 24 h is shown in Table II. The cumulative PDR of ^{13}C in urine from the coated colon targeted capsule (median 11.9 %, range 7.4–25.9 %) was in all collections lower ($p<0.05$) than the cumulative PDR of ^{15}N (median 81.6 %, range 76.6–86.8 %) in the same collection and the cumulative PDR of ^{13}C from the uncoated capsule for the same subject (median 73.1 %, range 64.0–77.9 %).

The median cumulative PDR at t=24 h of ^{15}N from the uncoated capsules in experiment 1 and 2 (73.7 % *versus*

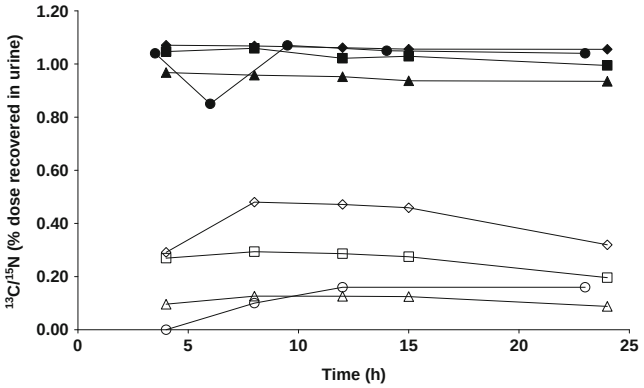

Fig. 2 Ratio $^{13}C/^{15}N$ (corrected for values at $t=0$) as percentage of the dose recovered in urine after intake of an uncoated ^{13}C-urea capsule (*closed symbols*) and a coated ^{13}C-urea capsule (*open symbols*). Similar symbols reflect the same subject. The time point is the time at which urine collection was finished.

81.6 %) showed 7.9 % absolute difference. The median cumulative PDR at $t=24$ h of ^{15}N and ^{13}C from the uncoated capsule in experiment 1 (73.7 % *versus* 73.1 %) showed 0.6 % absolute difference.

The median interindividual variation in cumulative PDR (3.4 %) was in the same range as the median interlabel variation (2.9 %). Median interday variation in cumulative PDR of ^{15}N was 9.7 %. Calculations of median variations were performed by combining the data from uncoated ^{13}C and ^{15}N-urea because we considered kinetics of both isotopes as equal after review of the results.

Breath Data

In Fig. 4 the breath ^{13}C exhalation data are shown, expressed as the cumulative PDR *versus* time curves over a time period of 15 h after intake of the coated capsule. All 4 subjects exhibited a significant exhalation of ^{13}C in breath varying from 54.5 to 81.5 %. These percentages represent the fermented fraction of ^{13}C-urea. The curves also indicate a lag time of >3 h. Figure 5 shows the fermented (breath)

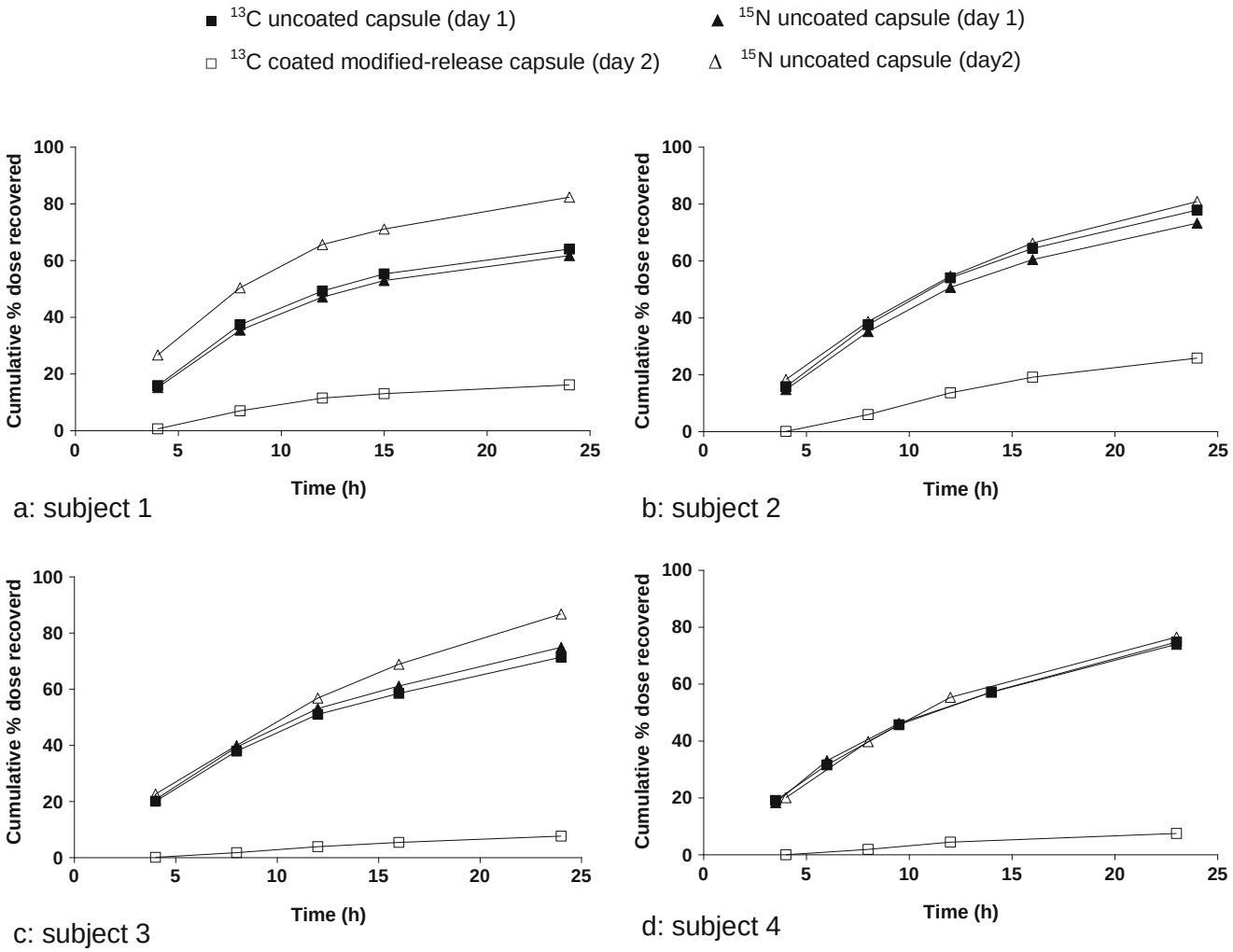

Fig. 3 Cumulative percentage non-fermented ^{13}C- and $^{15}N_2$-urea recovered from urine as a function of time. *black square* ^{13}C uncoated capsule (day 1), *black up-pointing triangle* ^{15}N uncoated capsule (day 1), *white square* ^{13}C coated modified-release capsule (day 2), *white up-pointing triangle* ^{15}N uncoated capsule (day 2).

Table II Cumulative PDR of ^{13}C and ^{15}N in Urine at t=24 h

Uncoated capsules				Coated capsule
Subject	^{15}N(exp 1)	^{15}N(exp 2)	^{13}C (exp 1)	^{13}C (exp 2)
1	61.8	82.3	64.0	16.1
2	73.3	80.9	77.9	25.9
3	74.9	86.8	71.4	7.6
4	74.0	76.6	74.8	7.4
Median	73.7	81.6	73.1	11.9
Mean	71.0	81.6	72.0	14.3
SD	6.2	4.2	5.9	8.7
CV	8.7	5.2	8.2	61.2
Median inter-individual variation (uncoated ^{13}C + ^{15}N)				3.4 %
Median interday variation (^{15}N)				9.7 %
Median interlabel variation (exp 1)				2.9 %

and non-fermented (urine) fractions of ^{13}C-urea recovered 15 h after intake of a coated capsule. Total recovery was large (>77 %), whereas the non-fermented fraction was limited (< 32 %).

Modeling

When $F_{non\text{-}fermented}$ calculated from a single urine-sample taken ≥12 h post dose was compared to $F_{non\text{-}fermented}$ calculated from the 24 h urine collection the absolute difference had a mean value of 8.6 % (95 % CI 5.5–11.7 %, p=0.068). The relationship between the $^{13}C/^{15}N$-ratios could be described by Eq. (1) obtained from the regression line.

$$\left(^{13}C/^{15}N\right)_{24h-collection} = \left(^{13}C/^{15}N\right)_{single-collection}/1,51\left(R^2 = 0.9977\right) \quad (1)$$

Using this equation the mean difference between the calculated $F_{non\text{-}fermented}$ from a single sample ≥12 h post dose and the 24 h urine collection was 0.1 % (95 %CI −0.3 to 0.5 %, p=0.67).

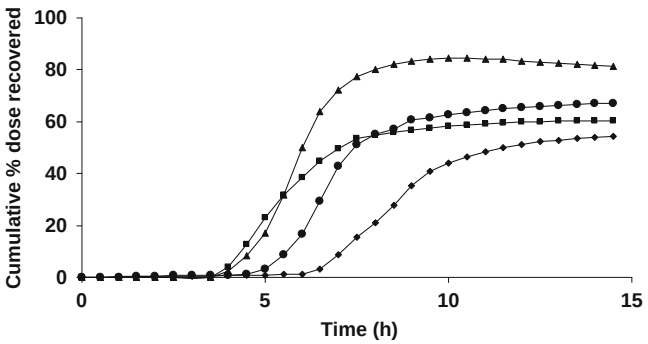

Fig. 4 Cumulative percentage recovery of ^{13}C in breath as percentage of the administered dose ^{13}C-urea (corrected for CO_2-retention) in all four subjects.

DISCUSSION

Kinetics of ^{13}C and $^{15}N_2$-Urea

The method of sample preparation and IRMS analysis proved to be reliable as shown by the spiking results. The accuracy was high with a recovery over 95 % and variation was low with a precision under 4 %. Furthermore the $^{13}C/^{15}N$-ratio was linear in a range of 0 to 1.0 (r^2=0.9999).

A prerequisite for the successful application of the dual stable isotope approach to determine local bioavailability is comparable kinetics of ^{13}C and $^{15}N_2$-urea. The so-called "isotope effect" (11,12) is the sum of differences in metabolism and physical properties (such as polarity, lipophilicity, protein binding) between the two different labeled compounds. Since urea is the end product of the nitrogen metabolism and therefore undergoes limited recycling, the kinetic isotope effect differentiating between ^{13}C and $^{15}N_2$-urea is expected to be absent. This hypothesis was tested in the first experiment. Two uncoated capsules containing 100 mg ^{13}C-urea and 50 mg $^{15}N_2$-urea respectively were taken simultaneously. Release of labelled urea will occur in the stomach and absorption of intact molecules into the systemic circulation will be fast. The mean of the $^{13}C/^{15}N$-ratio in urine for uncoated capsules shortly after administration was 1.01±0.06. This reflects the equimolar concentration in the urea distribution volume (UDV), pointing to comparable dissolution in the stomach, absorption, distribution and renal excretion. The interlabel variation appeared to be very limited as shown by the median recoveries from ^{15}N and ^{13}C in experiment 1 (73.7 % *versus* 73.1 %). This confirms the aforementioned hypothesis that both urea-isotopes have comparable kinetics and that the isotope effect is absent.

The cumulative PDR at t=24 h for ^{13}C or ^{15}N from uncoated capsules was high (61.8–86.8 %) as one expects for a small, water-soluble molecule, which is readily absorbed from the intestine. The amount not recovered can partly be explained by the so-called urea salvage. Intact absorbed urea diffuses from the systemic circulation back into the intestine where it may either be fermented (colon) or excreted via the feces. In earlier work we found this fraction to be 7.5 % at 12 h after oral administration (10). The major part of the non-recovered isotope label is probably still present in the urea distribution volume and will gradually be excreted in the urine afterwards, as the elimination half-life of urea is 7 h (12). This hypothesis is supported by two observations. First, the cumulative PDR-time curves in urine did not reach a plateau level within 24 h (Fig. 3). Second, fermentation was finished within 15 h (Fig. 4).

Since the "isotope effect" is absent for ^{13}C- and $^{15}N_2$-urea and the urea salvage is limited after release of urea in

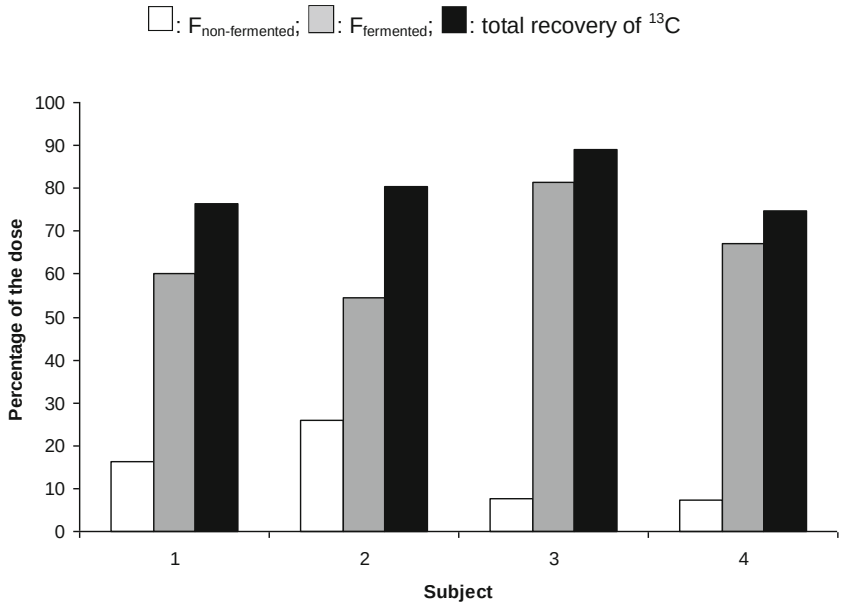

Fig. 5 Fractions fermented and non-fermented ^{13}C-urea after intake of a coated modified-release capsule; □: $F_{non\text{-}fermented}$; □: $F_{fermented}$; ■: total recovery of ^{13}C.

the stomach, $^{15}N_2$-urea excretion in urine collected over a certain time interval can serve as an internal standard correcting for day-to-day variation in urea kinetics. The fraction of non-fermented ^{13}C-urea after delivery in the colon may be quantified relative to this standard.

Dual-Label Stable Isotope Strategy

The second experiment aimed to give information on the release of ^{13}C-urea in the ileocolonic intestinal segment (urease-rich region). In an earlier proof-of-concept clinical study we showed that urea is fermented in the colon, which leads to reduced availability of intact ^{13}C-urea in blood and urine from a ColoPulse-capsule (9).

Urine and Breath Data

After concomitant administration of $^{15}N_2$-urea in an uncoated and ^{13}C-urea in a ColoPulse-capsule, the ^{13}C/^{15}N-ratio in urine became constant about 12 h after intake (Fig. 2). The ratio was much smaller than 1 in all subjects, which is explained by limited absorption of intact ^{13}C-urea from the ColoPulse-capsules in comparison to uncoated capsules (11.9 % *versus* 73.1 %). The fraction non-fermented of ^{13}C- and $^{15}N_2$-urea expressed as PDR per collected urine volume for each subject (Fig. 3) also illustrates the comparable kinetics of ^{13}C- and $^{15}N_2$-urea and the difference in segment of release of the uncoated and the ColoPulse-capsule.

The release profile of the ColoPulse-capsules obtained from the $^{13}CO_2$ response in breath (Fig. 4) is comparable to the one we found in the single dose, two-period crossover study (11). Median values found for the fermented fraction were 63.5 % in this study *versus* 69.2 % earlier. For the non-fermented fraction this was 14.8 % (urine) *versus* 16.0 % (blood). These results support our earlier finding that combining breath and urine data yields results comparable to combining breath and blood data.

The value of the ^{13}C/^{15}N-ratio in urine 12 h after intake becomes constant. This is explained by the release characteristics of the ColoPulse-capsule, which starts releasing its contents when the ileocolonic region is reached about 3 h after intake (Fig. 4).

The orocecal transit time (OCTT) is highly variable in healthy subjects and is dependent on the time interval between intake of the capsules and intake of food. The range of 3–5 h as observed for the lag time in the healthy volunteers in this study is in agreement with published OCTT data. Urease activity is related to the presence of bacterial load, which is normally absent in the mid small intestine, low in the distal small intestine and high in the colon. An exception is when bacterial overgrowth is present in the small intestine. However, this is not expected to occur in healthy subjects and therefore the subjects were not screened for this pathology. Furthermore no non-invasive, absolute diagnostic test is available for this purpose.

As might be expected, at 12 h all ^{13}C-urea has been released in the intestines and is absorbed or fermented or encapsulated in viscous feces. The curves of Fig. 4 indicate a lag time of >3 h. The combination of the delayed and the high ^{13}C response in breath proves that the capsule released its content in the urease-rich ileocolonic region.

The median cumulative PDR of ^{15}N released from the uncoated capsules in experiment 1 and 2 (73.7 % *versus* 81.6 %) showed 7.9 % absolute difference indicating day-to-day variation in urea kinetics. The median interday variation (9.7 %) appeared to be larger than the interlabel (2.9 %) and interindividual variation (3.4 %), supporting the proposal to apply $^{15}N_2$-urea released in the stomach as an internal standard to correct for day-to-day variations.

Single Urine Sample

When $F_{non\text{-}fermented}$ calculated from a single urine-sample taken ≥12 h post dose was compared to $F_{non\text{-}fermented}$ calculated from the 24 h urine collection the absolute difference in $F_{non\text{-}fermented}$ had a mean value of 8.6 % (p=0.07). This difference is caused by the excretion of ^{15}N-label during the first 4 h post dose when the coated ^{13}C-urea capsule still did not release its content.

We tried to find a reliable mathematical relationship between $^{13}C/^{15}N$-ratio in a single collection and in a 24 h-collection, taking into account the earlier start of ^{15}N-label excretion. This algorithm is to be used in future studies to be able to calculate the F non-fermented from a single urine sample. For each subject the mean $^{13}C/^{15}N$-ratio of the single urine collections ≥12 h post dose was plotted against the $^{13}C/^{15}N$-ratio in the 24 h urine collection. The obtained linear correlation coefficient was 0.9977 indicating a very strong relationship. This was confirmed by calculation of the $F_{non\text{-}fermented}$ both from a single sample (≥12 h post dose) and the 24 h urine collection. No difference could be detected between the $F_{non\text{-}fermented}$ obtained between these methods. The mean difference between these outcomes was only 0.1 %, (p=0.67) showing the validity of the model as used in this study.

The strong relationship between the $^{13}C/^{15}N$-ratio in a single urine sample collected ≥12 h post dose and that in the 24 h urine collection implies that the non-fermented fraction, needed to evaluate bioavailability of the content of a ColoPulse-capsule can be determined by analyzing the $^{13}C/^{15}N$-ratio in any urine sample obtained between 12 and 24 h after administration.

Increase of Study Power by One-Day Design

Heck *et al.* (13) reported already in 1979 that the application of stable isotope technology in bioavailability studies permits smaller group sizes by increased study power via elimination of day-to-day variation which is unavoidable in a two-day study design. To further evaluate the ColoPulse-technology, we calculated the difference in required group size when applying a one- or -two day study design by Eq. (2):

$$z_\beta = \frac{\delta}{\sqrt{\frac{2\sigma^2}{n}}} - z_{\alpha/2} \qquad (2)$$

We established a level of significance of 95 % (α=0.05, corresponding $z_{\alpha/2}$ = 1.96) and a power of 80 % (β=0.2, corresponding z_β = 0.84). We choose to be able to detect a difference (δ) in local bioavailability of 10 or 20 %. The population variance (σ) was estimated based on bioavailability data of our two-day (10) and one-day studies. As is shown in Table III, the group size (n) is smallest in the one-day study design and decreases more than proportional with a decrease in population variance.

Table III Sample Size Calculations for a Local Bioavailability Study of a Colon Drug Delivery System Applying Non-Invasive Stable Isotope Technology (α=0.05, β=0.2)

Reference	Detectable difference	Population variance	Required group size
Schellekens *et al.*, 2010 (10)	20 %	CV = 49 %	36
	10 %		144
This paper	20 %	CV = 18 %	5
	10 %		21

Together with the elimination of blood samples, the reduction of breath samples by performing a study in 1 day and the absence of day-to-day variation in urea kinetics add additional advantages to the earlier proposed study design using coated and uncoated capsules containing ^{13}C-urea on different days (11). We will further investigate this approach in a clinical study to evaluate the release profile and local bioavailability of colon-specific tablets in both healthy subjects and patients with Crohn's disease.

Another part in clinical development of a new drug delivery system or drug product is determination of bioequivalence when comparable devices or products are already available. The dual-label stable Isotope strategy was not intended for studies with active substances, because drug specific characteristics cannot be tested. However the principle can be used for testing bioequivalence of colon-specific drug delivery devices. In that case the study has to be performed on two different days, but all the other mentioned advantages of this approach are still applicable including the absence of day to day variation. The single challenge is the availability of a comparator drug delivery device containing ^{13}C-urea.

CONCLUSION

Application of a dual-label stable isotope strategy of $^{15}N_2$- and ^{13}C-urea is suitable for the evaluation of bioavailability of colon-specific drug delivery systems. Since both isotopes can be taken at the same time, day-to-day variation in urea kinetics is eliminated and study power is increased.

Compared with the conventional two-period study design, our approach reduces clinical study costs by a decrease in study run through time (one period instead of two) and in sample-load by omitting blood-samples, reducing breath samples by 50 % and only taking one urine sample. With this feasibility study we showed that combination of breath and a single urine sample provides sufficient information to

assess ColoPulse-capsules *in vivo* without radioactive exposure in a non-invasive, low-cost study design.

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