

Effect of Casilan® on ¹³C-caffeine metabolism in overnight-fasted healthy Nigerian children

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ABSTRACT

Objective: To determine the effect of Casilan® on ¹³C-caffeine metabolism in healthy Nigerian children. **Materials and Methods:** Twelve healthy Nigerian children (male: six, female: six) aged 3–8 years were studied on three occasions. After an overnight fast, the children were studied after ingesting Casilan® only (Week 1). They were restudied after ingesting 3 mg/kg of labeled caffeine only (Week 2), and further re-studied after ingesting both Casilan® and labeled caffeine (Week 3). Breath samples were collected by blowing via a straw into an exentainer bottle. The cumulative percentage of ¹³C-caffeine exhaled as ¹³CO₂ was measured over 2 h. **Results:** The time courses of ¹³C-enrichments in exhaled CO₂ for all the children, after they had ingested labeled caffeine only and after they had ingested both Casilan® and labeled caffeine, were identical. There was a gradual rise and peak of the enrichments at about 60–75 min, followed by a gradual fall (II) or a plateau (III). Contrarily, the time course of ¹³C-enrichments for all the children was consistently low and stable after they had ingested Casilan® only (I). The mean values of cumulative percent ¹³C-doses recovered in the CO₂ exhaled over a 2-h period, after ingesting labeled caffeine only (8.59 ± 1.10 δ%/mg) and after ingesting both Casilan® and labeled caffeine (8.58 ± 1.33 δ%/mg), were identical, with no statistically significant difference (*P* = 0.972). This suggests that Casilan® did not affect the CYP1A2 metabolic pathway. **Conclusions:** Casilan® is a safe, reliable and quantitative food supplement for overnight-fasted children undergoing caffeine breath test.

Key words: CYP1A2 metabolizing enzyme, food supplement, healthy children, labeled caffeine

INTRODUCTION

The concept that blood, urine and other body fluids and tissues can be collected and analyzed to yield information for diagnosis of disease states or to monitor disease progression and/or therapy is the foundation of modern medicine.^[1] However, advanced tracer technology with application

of stable isotopes in humans has enabled the noninvasive observation of metabolic processes and the assessment of enzyme activities, organ functions and transport processes *in vivo*.^[2] The stable isotope technique, particularly the use of ¹³C-breath tests for diagnostic purposes, has remarkably enriched the gastroenterological diagnostic spectrum and allowed the study of effect of diseases on drug metabolism and drug interaction in children.^[3]

The majority of ¹³C-breath tests performed in children and adults involved oral administration of ¹³C-labeled substrate to the subject on an empty stomach.^[4] The precision of CO₂ breath test, using ¹³C-substrate, is affected by natural fluctuations in the ratio of ¹³C/¹²C in the expired CO₂.^[5] Most of the natural food substances (carbohydrates, proteins and lipids) and their products contain a varying low proportion of ¹³C, which

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may increase $^{13}\text{CO}_2$ in breath tests.^[4] The natural fluctuations of $^{13}\text{C}/^{12}\text{C}$ ratio are increased if the subject had eaten either immediately before or during the breath test.^[5] Also, a meal before or during the breath test would increase the basal metabolic rate, resulting in an overestimation of the predicted resting carbon dioxide production rate ($V\text{CO}_2$).

Caffeine breath test (CBT) involves the use of a nonradioactive stable isotope (^{13}C on the 3-methyl group of caffeine). The labeled caffeine is given orally and undergoes 3-N-demethylation, which is a cytochrome *P*-450-dependent reaction (CYP1A2). After N-demethylation, the labeled methyl group enters the carbon pool as it is converted to formaldehyde, formate and bicarbonate. The bicarbonate is exhaled as carbon dioxide and the exhaled labeled $^{13}\text{CO}_2$ correlates with CYP1A2 activity.^[3]

The subjects for ^{13}C -breath tests are investigated at rest in a sitting position.^[4] Increased physical activity during the breath test would affect endogenous CO_2 production.^[6] Breath samples are collected over a 2–6 h period, depending on the ^{13}C -substrate administered. Children may rarely be able to sit for this length of time and may rarely fast for the duration of the test; thus, their energy expenditure may likely be above the resting values. Using the resting $V\text{CO}_2$ to calculate the proportion of ^{13}C -substrate recovered in breath (percentage dose recovered [PDR]), which is the area under the curve of the rate of appearance of labeled CO_2 (PDR/h) against time, may underestimate the true recovery of ^{13}C in breath CO_2 .^[7]

PDR is a function of breath CO_2 enrichment, $V\text{CO}_2$ and the amount and enrichment of the ingested ^{13}C -substrate.^[7] A resting value of $V\text{CO}_2$ is usually assumed in many ^{13}C -breath tests. A predicted value of resting $V\text{CO}_2$ of 300 mmol/(BSA·h),^[8] where body surface area (BSA) = $0.024265 \cdot W^{0.5378} \cdot H^{0.3964}$ (W means body weight in kg and H is the body height in cm),^[9] is widely used in many ^{13}C -breath tests. The BSA equation takes into account the variation of $V\text{CO}_2$ with body size and allows the same value to be assumed in subjects of all ages and size. Resting $V\text{CO}_2$ was also predicted from basal metabolic rate, which was a function of age, gender, height and weight using the Schofield equations^[10] and assuming a respiratory quotient (RQ) of 0.85.^[11] However, most researchers found this method to be inaccurate for young children when compared with the method of Shreeve *et al.*^[8]

Slater *et al.*^[12] have raised concern for the accuracy and reliability of the available methods of estimating the predicted values of resting $V\text{CO}_2$ in children. They estimated the total (nonresting) $V\text{CO}_2$ in adults and children during ^{13}C -breath tests using smoothed and raw heart rates with two nonlinear functions (sigmoid and third-order polynomial equations [$x = [a/(1 + e^{-(y-b)/c})] + d$], where x is $V\text{CO}_2$, y is heart rate and a , b , c and d are constants: a is the amplitude, b is the center, c is the width of the curve and d is an offset, theoretically equivalent

to resting metabolic rate). Slater *et al.*^[12] reported that their method was more accurate and precise in estimating the total $V\text{CO}_2$ in adults and children thus improving the accuracy of calculated PDR in ^{13}C -breath tests. However, this method has not been validated by other studies and is yet to gain popularity in ^{13}C -breath tests in children. Moreover, facilities for the heart rate calibration procedure may not be readily available in resource-poor countries where ^{13}C -breath tests may be useful as noninvasive methods for pediatric pharmacological research in malnourished children.

Previous studies in children involving the use of CBT have shown that children tolerated overnight fasting and the 2-h period for breath samples collection.^[3] Also, physical activities were minimized by engaging the children in fun activities and collecting samples in a sitting position.^[3] These further explain the reasons for the popularity of the methods of Shreeve *et al.*^[8] and Schofield^[10] for estimating total $V\text{CO}_2$ in CBT in children.

Nutritional deficiency is a major problem of malnourished children; thus, making these children fast over the 2-h period for CBT may not be feasible, for obvious ethical reasons. A low carbohydrate diet is suggested when fasting is not feasible in the pediatric subjects.^[7] Casilan® is a bland high-quality protein powder derived from milk. It is very low in fat and carbohydrate (both less than 1%).^[13] It contains no added sugar, artificial sweeteners, colors, flavors or preservatives, making it an ideal supplement to many drinks and foods to boost the protein content without altering the flavor. A knowledge gap exists on the effect of food supplement on the proportion of ^{13}C -substrate recovered in breath CO_2 during CBT study in children.

The use of Casilan® as a food supplement in ^{13}C -CBT to address the unethical issues surrounding fasting of pediatric subjects has not yet been determined. The aim of this study was to determine the effect of Casilan® as a food supplement on ^{13}C -caffeine metabolism in overnight-fasted healthy children in Nigeria.

MATERIALS AND METHODS

Subjects

Twelve healthy children (six male, six female) were recruited at the general pediatric outpatient clinic, General Hospital, Ikorodu in Lagos, Nigeria. A sample size of 12 subjects has a power of 90%, assuming a difference of 2% in the mean \pm SD score of the 2-h percentage cumulative ^{13}C from the baseline to 1 week of re-study, and the sample size is appropriate for this study at the 5% level of significance.^[3] Subjects were included if they were less than 12 years old and clinically fit after medical examination. The subjects were excluded if they

were on regular medication, had hearing impairment, cerebral palsy, elevated blood pressure, abnormal renal or liver function or reported any cardiovascular, respiratory or gastrointestinal disease. The Ethics Committee of the Lagos State Health Service Commission approved the study. The parent of each subject ≤ 6 years gave written informed consent for their child to participate in the study. Each subject above 6 years gave assent and their parents as well gave written informed consent to participate in the study.

Study design

An appointment was given to each of the subjects for 08:00 h on different days between Mondays and Fridays. Subjects abstained from caffeine-containing products for 24 h before the study was commenced. They also fasted overnight and did not take anything orally on the appointment days. On the days of the study, each subject was attended to in a private consulting room in the presence of one of the parents. Body weight was measured (to 0.1 kg) using Seca scales (model 707; Seca Ltd., Birmingham, UK), with the subjects wearing only underpants. Height was measured (to within 1 mm) using a Holtain stadiometer (Holtain, Crymych, Dyfed, UK), with the subjects barefoot. Subjects were considered normal if their weight for age and weight for height z-score were ± 3 SD of the WHO child growth chart standards.^[14,15] Vital signs including blood pressure, heart and respiratory rates and temperature were recorded and monitored throughout the study.

The study involved subjects sitting quietly for half an hour before and throughout the CBT in order to minimize physical activities that can influence endogenous CO₂ production and may affect CBT results. In a previous CBT study in adults, normal healthy subjects were re-studied after approximately 1 week.^[16] Our subjects were therefore studied on three occasions at a weekly interval. The first study involved subjects ingesting Casilan® only (study I), made into a cream, at 09:00 h, calculated as one-twelfth of the Recommended Dietary Allowance of calories (RDA: children 3–6 year old [90 kcal/kg], children 7–10 years old [70 kcal/kg]).^[17] Paired breath samples were collected during normal expiration at -20, -10 and -1 min prior to Casilan® (predose samples) and after Casilan® ingestion (postdose samples) at 15-min intervals over a 2-h period. Breath samples were collected by getting each child to blow via a straw into an exentainer bottle.

The second study involved subjects ingesting 3 mg/kg of [3-methyl-¹³C] caffeine (99% ¹³C) (study II) at 09:00 h. The caffeine was obtained in powder form from Cambridge Isotope Laboratories (Cambridge, MA, USA). Each sample of the caffeine was dissolved in 10 mL of sterile water and mixed with sugar-free squash to mask its bitter taste, followed by a 20 mL water wash of the container. The quantity of caffeine consumed was approximately equivalent to the amount present

in a coke drink. Paired breath samples were collected during normal expiration at -20, -10 and -1 min prior to caffeine (predose samples) and after caffeine ingestion (postdose samples) at 15-min intervals over a 2-h period. Subjects were observed for signs of caffeine toxicity with detailed symptom assessment, while blood pressure and respiratory and heart rates were monitored every half an hour during testing.

The third study involved subjects ingesting Casilan® cream, calculated as one-twelfth of the RDA of calories,^[18] about half an hour prior to caffeine administration (study III). Caffeine administration and breath sample collection were according to the protocol explained earlier. The breath samples were stored in the exentainer bottles for 12 weeks before being couriered to the Clinical Physiology Laboratory of the Medical School in Derby, University of Nottingham, for analysis.

Analytical methods

¹³C enrichment of breath CO₂ was determined by continuous-flow isotope ratio mass spectrometry^[19] using a SerCon-Automated Breath Carbon Analyser (CF-IRMS) (Europa Hydra; SerCon Ltd, Crewe, UK). Ten milliliter aliquots of breath samples were injected automatically into the analyzer where they were dried and resolved from nitrogen and oxygen by gas chromatography. The purified CO₂ was then passed, using helium as a carrier, into an electron impact ion source of the isotope ratio mass spectrometer. The ion beams at 44, 45 and 46 m/z were monitored continuously and used to evaluate ¹³C enrichment of CO₂, with reference to a 2% CO₂/98% N₂ gas supply, which had been calibrated against a bicarbonate standard of known ¹³C enrichment. Duplicate analyses of each breath sample were performed.

Calculations and statistical analysis

¹³C-enrichment of exhaled CO₂ was converted from delta units to atom percent using the accepted atom fraction of the international bicarbonate standard.^[18] ¹³C-enrichment was expressed as atom % ¹³C excess by subtracting the average predose enrichment from each postdose measurement. Cumulative ¹³CO₂ output was calculated by averaging the measured ¹³C-enrichment of the eight breath samples taken during the first 2 h following administration of the ¹³C-caffeine dose and multiplying this by the average output CO₂ (V/CO_2) over this period (assumed to be 300 mmol/[BSA · h]).^[8,9] This was expressed as a percentage of the caffeine dose.

The subjects were compared after ingesting Casilan® or labeled caffeine only, and after ingesting both Casilan® and caffeine by using a Student's paired *t*-test at a $P < 0.05$ significant level. Spearman's rank correlation, r_s , was used to determine the correlation between age, weight or height of the children, and the cumulative percent ¹³C-caffeined dose exhaled in 2 h carbon dioxide in each study.

RESULTS

The average ^{13}C -enrichment for the 12 children was calculated at each time the breath samples were collected over a 2-h study period. The average ^{13}C -enrichments were determined for the children after they had ingested Casilan® only (study I), after they had ingested labeled caffeine only (study II) and after they had also ingested both Casilan® and labeled caffeine (study III). The time courses of ^{13}C enrichments for the children, at different stages of the study (I-III), are shown in Figure 1. The curves differed according to the intake of a food supplement, i.e. the children had either ingested or not ingested Casilan® (a food supplement). After ingesting Casilan® only, the ^{13}C enrichments in the exhaled CO_2 were consistently low, fluctuating between $\pm 10^{-3}$ delta%, and yielded almost a plateau line [I in Figure 1]. This suggests a low level of ^{13}C in the Casilan®. The ^{13}C enrichments in the exhaled CO_2 of the children were identical after they had ingested labeled caffeine only and after they had ingested both Casilan® and labeled caffeine. The ^{13}C -enrichments followed a typical time course for CBT. The values increased gradually, reaching their peak values at about 60–75 min [II and III in Figure 1], followed by a gradual fall [II in Figure 1]. However, the ^{13}C enrichments approached a plateau after attaining the peak value in children who ingested both Casilan® and caffeine [III of Figure 1].

The demographics and caffeine dose exhaled as $^{13}\text{CO}_2$ over a 2-h period for each child, at different stages of the study (I-III), are shown in Table 1. The percentage caffeine dose in the cumulative 2-h $^{13}\text{CO}_2$ was lower in the children after they

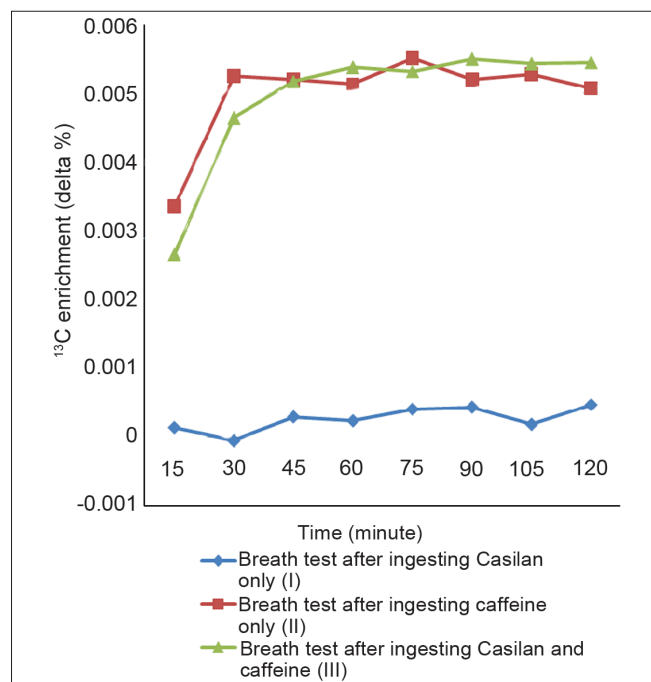


Figure 1: Time course of ^{13}C enrichment for normal healthy Nigerian children after ingesting Casilan® or labeled caffeine only, and after ingesting both Casilan® and caffeine

ingested Casilan® only (I) compared with after they ingested labeled caffeine only (II) and after they ingested both Casilan® and labeled caffeine (III). The percentage caffeine dose in the cumulative 2-h $^{13}\text{CO}_2$ was higher in seven children (four male, three female) after they had ingested both Casilan® and labeled caffeine compared with after they had ingested caffeine only. However, the remaining five children had contrasting results.

The CBT of the children, after they ingested Casilan® or labeled caffeine only and after they ingested both Casilan® and caffeine, is presented in Figure 2. Data were expressed as mean \pm SD. The mean values of the percentage caffeine dose exhaled over 2 h were identical after the children had ingested caffeine only ($8.59 \pm 1.10 \delta\%/mg$) and after they had ingested both Casilan® and labeled caffeine ($8.58 \pm 1.33 \delta\%/mg$). There was no statistically significant difference in the mean values ($P = 0.972$) [Table 1]. However, after the children had ingested Casilan® only, the mean value of the percentage caffeine dose exhaled over 2 h ($0.58 \pm 1.39 \delta\%/mg$) was significantly lower than the values after they had ingested caffeine only ($P = 0.000$) and after they had ingested both Casilan® and labeled caffeine ($P = 0.000$). The cumulative $^{13}\text{CO}_2$ exhaled over a 2-h period is dependent on the 3-N-demethylation of caffeine. The very low level observed after the children had ingested Casilan® only and the identically high levels observed after the children had ingested caffeine only and after they had ingested both Casilan® and labeled caffeine would suggest that Casilan®, as a food supplement, did not significantly affect the CYP1A2 metabolic pathway.

After ingesting Casilan® and labeled caffeine, the percentage caffeine doses exhaled over 2 h by seven children were increased compared with after they ingested labeled caffeine only. Their mean values were $8.97 \pm 1.00 \delta\%/mg$ and $8.21 \pm 0.95 \delta\%/mg$, respectively, and the difference in the mean values was not statistically significant ($P = 0.054$). However, the percentage caffeine doses exhaled over 2 h by the remaining

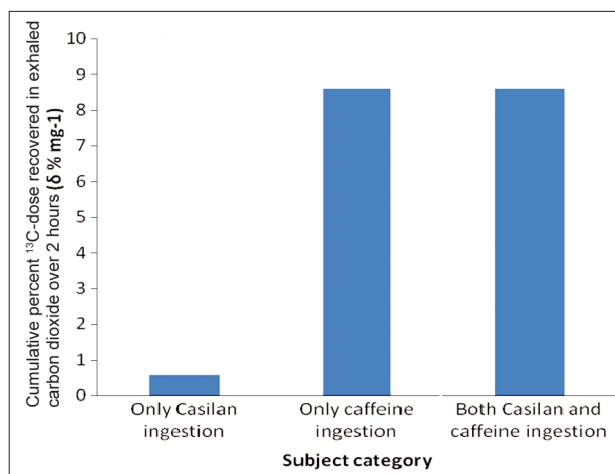


Figure 2: Caffeine breath test following the ingestion of Casilan®, labeled caffeine and both Casilan® and labeled caffeine

Table 1: Clinical details and average percentage ¹³C-caffeine in the cumulative 2 hour CO₂ output of healthy subjects without ingesting and after ingesting Casilan®

Subject	Gender	Age (years)	Weight (kg)	Height (cm)	Weight-for-age z-score	Weight-for-height z-score	Resting VCO ₂ over the 2-h study period (mmol/h)	Average percent ¹³ CO ₂ exhaled after ingesting Casilan® only	Cumulative percent ¹³ C-dose (caffeine) recovered in the 2-h CO ₂ exhaled after ingesting Casilan® only (δ %/mg)	Average percent ¹³ CO ₂ exhaled after ingesting caffeine only	Cumulative percent ¹³ C-dose (caffeine) recovered in the 2-h CO ₂ exhaled after ingesting caffeine only (δ %/mg)	Average percent ¹³ CO ₂ exhaled after ingesting Casilan® and caffeine	Cumulative percent ¹³ C-dose (caffeine) recovered in the 2-h CO ₂ exhaled after ingesting Casilan® and caffeine (δ %/mg)
I	F	7.25	22	119	1 SD	2 SD	176.36	-0.00579	-1.02	0.03996	7.04	0.04180	7.37
II	F	5.00	20	105	1 SD	2 SD	195.10	0.01070	2.09	0.04120	8.04	0.04224	8.24
III	M	3.67	13	94	-1 SD	-1 SD	256.97	-0.00213	-0.55	0.03020	7.76	0.03350	8.61
IV	M	5.25	17	110	1 SD	-1 SD	209.01	0.00567	1.18	0.03723	7.78	0.04916	10.27
V	M	3.50	13	99	-1 SD	-2 SD	286.46	0.01106	3.17	0.02781	7.97	0.03100	8.88
VI	F	6.08	19	118	-1 SD	-1 SD	191.47	-0.00135	-0.26	0.05216	9.99	0.04132	7.91
VII	F	6.08	21	120	1 SD	-1 SD	180.23	-0.00228	-0.41	0.0584	10.53	0.05523	9.95
VIII	M	4.20	17	108	1 SD	2 SD	106.87	0.01179	1.26	0.07879	8.42	0.09245	9.88
IX	M	7.00	21	122	0 SD	0 SD	179.06	0.01161	2.08	0.05378	9.63	0.05144	9.21
X	F	5.25	18	116	1 SD	1 SD	198.46	-0.00443	-0.88	0.04651	9.23	0.04565	9.06
XI	F	6.50	20	120	1 SD	0 SD	185.02	-0.00351	-0.65	0.04291	7.94	0.04097	7.58
XII	M	7.83	21	119	1 SD	-1 SD	180.83	0.00521	0.94	0.04388	7.93	0.03110	5.62
Mean ± SD									0.58 ± 1.39 ^a		8.59 ± 1.11 ^{a,b}		8.58 ± 1.33 ^{a,b}

^aThere was a significant difference in the calculated mean cumulative percent ¹³C-doses (caffeine) recovered in the 2-h CO₂ exhaled in the subjects after they ingested Casilan® or caffeine only, and after they ingested both Casilan® and caffeine (*P* = 0.000). ^bThere was no significant difference in the calculated mean cumulative percent ¹³C-doses (caffeine) recovered in the 2-h CO₂ exhaled in the subjects after they ingested caffeine only and after they ingested both Casilan® and caffeine (*P* = 0.972)

five children were decreased after ingesting Casilan® and labeled caffeine compared with after they ingested labeled caffeine only. Their mean values were 8.02 ± 1.63 $\delta\%/mg$ and 9.12 ± 1.18 $\delta\%/mg$, respectively. The difference in the mean values was also not statistically significant ($P = 0.069$). These results further suggest that Casilan® has no significant effect on CYP1A2 metabolic activities.

There was no significant correlation between age ($r = -0.396$, $P = 0.202$), weight ($r = -0.287$, $P = 0.366$), height ($r = -0.309$, $P = 0.329$) and cumulative percent of labeled caffeine dose recovered in the 2-h CO_2 exhaled after ingesting Casilan® only. No significant correlation was also observed between age ($r = -0.018$, $P = 0.957$), weight ($r = 0.117$, $P = 0.718$), height ($r = 0.330$, $P = 0.295$) and cumulative percent of labeled caffeine dose recovered in the 2-h CO_2 exhaled after ingesting labeled caffeine only. Similarly, no significant correlation was observed between age ($r = 0.453$, $P = 0.140$), weight ($r = -0.372$, $P = 0.234$), height ($r = -0.109$, $P = 0.736$) and cumulative percent of labeled caffeine dose recovered in the 2-h CO_2 exhaled after ingesting both Casilan® and labeled caffeine.

DISCUSSION

This study is the first of its kind to evaluate the effect of Casilan®, a food supplement, on caffeine metabolism. It is part of a large study aimed at using a noninvasive method, particularly the CBT, to assess the effect of malnutrition on caffeine metabolism. Fasting is very essential in CBT studies, but may not be ethically feasible in malnourished children. There is an unfulfilled clinical need for a food supplement to be administered to malnourished children undergoing CBT. There has never been a search for a safe, reliable and quantitative food supplement that will yield reliable CBT data in malnourished children probably because CBT has not been applied to the study of effect of malnutrition on drug disposition in children.^[20]

The results from the present study showed that Casilan® is a safe and reliable food supplement for children undergoing CBT. Our data indicate that (1) Casilan® was administered easily and safely to children, (2) caffeine metabolism was not significantly influenced by Casilan® ingestion as demonstrated by the pattern of time course of ^{13}C enrichments in breath CO_2 after the children ingested either Casilan® or labeled caffeine and after they ingested both Casilan® and labeled caffeine, (3) the ^{13}C enrichment, after the children ingested Casilan® only, remained stable at very low levels, yielding almost a flat plot, (4) peak values of the ^{13}C enrichment after the children ingested caffeine only and after they ingested both Casilan® and labeled caffeine were attainable at an average time of 60–75 min, followed by a gradual decline or a sustained plateau and (5) the mean value of the percentage

cumulative caffeine doses exhaled by the children over 2 h after they ingested both Casilan® and labeled caffeine was identical to the mean value obtained after they ingested labeled caffeine only, and the difference in the mean values was not statistically significant.

The time courses of caffeine metabolism after the children had ingested labeled caffeine only and after they had ingested both Casilan® and caffeine are in general agreement with previously published breath test studies in children.^[21] Park *et al.*^[16] have shown that the time to peak plasma caffeine concentrations as well as peak caffeine level after oral administration was 1 h for normal adult control patients. Different developmental patterns have been identified for CYP-450 activities in children. The fetal liver has between 30% and 60% of adult total cytochrome *P*-450 values, and approaches adults values by 10 years of age.^[22] Given the age range (3–8 years) of the children studied, their CYP1A2 quantity and activities are likely to be slightly lower than those of the adults. This may explain the slight variation in time (60–75 min) to peak labeled caffeine levels observed in our study. Studies have also shown that optimal correlation between the plasma clearance of caffeine in infants, older children and adults, and the cumulative excretion of $^{13}CO_2$ was at 2 h.^[23] The fact that the time to peak caffeine levels was not significantly different after the children had ingested labeled caffeine only and after they had ingested both Casilan® and caffeine may suggest that Casilan® did not alter the CYP1A2 activity involved in the hepatic metabolism of caffeine.

Carbohydrate diets are rich in ^{13}C ^[4] and are likely to increase the exhaled $^{13}CO_2$ during CBT. Casilan®, being very low in fat and carbohydrate, is unlikely to have contributed to the slightly higher levels of ^{13}C enrichment and the cumulative $^{13}CO_2$ exhaled over the 2-h study period observed in the seven children after they ingested Casilan® and caffeine, compared with after they ingested caffeine only. Ingestion of Casilan® will likely increase the basal metabolic rate with a corresponding increase in endogenous $^{13}CO_2$ production in the subjects. Given the low quantity of Casilan® (one-twelfth of the RDA) administered to the subjects, the endogenous $^{13}CO_2$ production is likely to be minimal and very unlikely to influence the levels of ^{13}C -enrichments and the cumulative $^{13}CO_2$ exhaled after the subjects ingested Casilan®.

The ^{13}C recovered in the exhaled $^{13}CO_2$ of five children, over the 2-h study period, was slightly lower after they ingested Casilan® and caffeine than after they ingested caffeine only. Compared with the seven children with contrasting results, the difference in the ^{13}C recovery may be explained by physiological effects. The lower ^{13}C recovery in the five children may indicate that ^{13}C was partly lost via a route other than the pulmonary route. It has been previously reported that 1–2% of total CO_2 excretion was lost across the skin.^[24]

The imperceptible lost may vary from one child to another. The recovery of ^{13}C may also be reduced by ^{13}C fixation in the body pools and constituents with a slow turnover such as bone, skeletal tissue and fat, and, to a lesser part, excretion via urine and feces.^[25] The latter was estimated to be smaller than 6% in bulls.^[26]

Rigorous stability testing has not yet been performed with the CBT, particularly in tropical African countries. However, we can extrapolate the stability data on ^{13}C -urea breath samples,^[27] which are essentially the same as the breath samples in this study, suggesting that CBT samples from our subjects are similarly stable. The present study is likely the first of its kind to extend CBT to children in Africa. CBT samples were collected and stored for about 12 weeks before being transported to the United Kingdom for analysis. The results from our study are reliable and suggest that the CBT samples were stable over the period of storage in Nigeria. This is however a significant advantage of CBT over other quantitative assays.

Previous CBT in children and adults have shown that caffeine metabolism, expressed as a 2-h cumulative exhalation of $^{13}\text{CO}_2$, is not influenced by age^[28,29] and gender^[28,29] in older children, similar to the age group we studied. However, we were unable to draw a firm conclusion with regard to the influence of age and gender on caffeine metabolism in children after they ingested Casilan® only and after they ingested both Casilan® and labeled caffeine due to the few subjects involved in the study.

Intra- and interindividual variations in CYP1A2 activity have been reported.^[30] About 35–75% of the interindividual variability in CYP1A2 activity is due to genetic factors.^[31] The interindividual differences are likely to influence the metabolism of ^{13}C as well as explain the variation in the cumulative ^{13}C -dose recovered in the 2-h CO_2 exhaled by each subject. It is hoped that future studies would explore the genetic roles of CYP1A2 in ^{13}C metabolism. However, the use of the same individual as subjects and controls in this study has eliminated possible bias from interindividual variation in CYP1A2 activity that characterized previous CBT. Similarly, studying the subjects at the same time of the day (09:00 h) also has helped eliminate bias due to intraindividual genetic variation.

In conclusion, the present study confirms that Casilan® is a safe, simple and quantitative food supplement for overnight-fasted children undergoing oral CBT. The reliability of the yielded data suggests that Casilan® may be used to address unethical issues surrounding fasting of nutritionally deficient children undergoing CBT. This pilot study, in a few number of children, is considered as the basis for validation in a larger study.

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