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Maturation of Caffeine N-Demethylation in Infancy: A Study Using the $^{13}CO_2$ Breath Test

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ABSTRACT. Four premature neonates and eight infants 1–19 months old received caffeine for apnea. The usual morning oral dose was substituted by 1,3,7 ^{13}C-trimethylxanthine (^{13}C-tri CAF) as the citrate salt. Five breath samples were collected the day before (day 1) and the day of ^{13}C-tri CAF administration (day 2). Plasma (after each breath collection) and urine were collected on day 2. ^{13}C-CO_2 exhalation was determined by isotope ratio mass spectrometry. Caffeine and its metabolites were measured using high-pressure liquid chromatography. Assessment of the labeled CO_2 in the breath revealed no detectable ^{13}C-tri CAF N-demethylation activity in infants before 45 wk postconceptional age. However, demethylation (as urinary metabolites) has been detected before that age. Two-, 4-, and 6-h cumulative excretion of ^{13}C-tri CAF as ^{13}C-CO_2 increased with postnatal age and correlated with caffeine plasma clearance ($r = 0.840$, $p < 0.01$). These results were consistent with those obtained for urinary metabolites. In one infant (19 months old) the cumulative excretion of ^{13}C-CO_2 while crying was 65% of the value observed during quiet breathing. The measurement of caffeine demethylation using the caffeine CO_2 breath test is feasible in infants and is a safe and noninvasive method to determine age related changes in P450I-dependent N-demethylase activity. (*Pediatr Res* 23: 632–636, 1988)

Abbreviations

CAF, caffeine (trimethylxanthine)
^{13}C-tri CAF, 1,3,7 ^{13}C3-trimethylxanthine

For several years, CAF has been used for the treatment and prevention of apnea in premature newborn infants (1, 2). More recently it has been used in young infants with near-miss sudden infant death syndrome (3) and with the Pierre Robin syndrome (4). The CAF dose regimen depends on the variation of pharmacokinetic parameters with maturation. Elsewhere we described the pattern of this variation (5). The important interindividual variability of CAF kinetic parameters complicates the design of dose regimen guidelines implying that individual drug monitoring is necessary. The variation of CAF pharmacokinetic parameters with age is a consequence of the maturation of CAF metabolism. Our study was designed to better characterize the maturation of the main CAF metabolic pathways involving N-demethylation in infancy and to develop a noninvasive method in children for metabolic studies and CAF monitoring using stable isotope labeling.

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MATERIALS AND METHODS

The clinical data are presented in Table 1. Twelve children were selected. A preliminary study was performed in one nonsmoking healthy adult weighing 75.5 kg. The pediatric group consisted of four newborn infants (all prematurely born) and eight older infants (three prematurely born); seven infants had Pierre Robin syndrome. They were respectively 21 ± 1.5 (SD) (range 19–22) days and 30–588 days old. The gestational ages, determined by the menstrual history of the mother and by the physical examination of the infants using the criteria of Amiel-Tison (6) were, respectively, 32 ± 0.9 (SD) (range 31–33) and 37 ± 3.1 (SD) (range 31–41) wk. All subjects received CAF citrate solution (25 mg/ml) orally for the treatment and prevention of apnea. The dose regimen was determined initially using individual drug monitoring. The children were treated for at least three drug half-lives (5–35 days) to achieve steady-state. One usual morning oral maintenance dose was then substituted by ^{13}C-tri CAF citrate. The adult received a single oral dose. Patient G was examined twice and patient H four times at different postnatal ages. None of the children had a history of hepatic disease and none was receiving any drug known to affect methylxanthine elimination. All patients were born of nonsmoking mothers. Informed consent was obtained from the parents and the study was approved by the Institut National de la Santé et de la Recherche Médicale (INSERM).

^{13}C-tri CAF (98% ^{13}C labeled) and ^{13}C-CO_2 (90% ^{13}C) were obtained from CEA (Commissariat à l'Energie Atomique, Service des Molécules Marquées, Gif sur Yvette, France).

For the CO_2 breath test the patients were breathing spontaneously. The expired gas was collected over a 1-min period using a face mask (Rendell Baker size 0 or 1 according to the patient size) fitted via a small dead volume (4 ml) two-way valve (Ambu Paedi, M.M.S.–SANOFI, France) into a 3-liter latex bag. The patients were allowed to breathe for 1 min into the face mask before the gas collection. The volume of expired gas was measured with a pneumotachograph (Gould Godart, M.M.S.–SANOFI, France) connected to a flow transducer (Fleisch 00) inserted between the valve and the latex bag. Aliquots of 450 ml were transferred to evacuated glass flasks for analysis. Five breath samples were collected the day before labeled CAF administration (day 1), to determine the baseline for endogenous ^{13}C-CO_2

Table 1. *Clinical data*

Patient	Gender	Gestational age (wk)	Postnatal age (days)	Wt (g)
A	M	33	19	2310
B	M	32	22	2110
C	M	31	22	1460
D	M	31	22	1850
E	M	31	30	2400
F*	M	40	33	3780
G(I)*	M	41	52	4395
H(I)*	F	38	54	3620
I*	M	39	174	5070
J*	M	36	273	6370
K*	M	37	434	9100
L*	M	36	588	9100
G(II)	M	41	92	5250
H(II)	F	38	112	4580
H(III)	F	38	196	5670
H(IV)	F	38	252	5840

* These infants presented with Pierre Robin syndrome.

production, and again on the day of labeled CAF administration (day 2) according to the following protocol—before the morning dose, at 2, 4, and 6 h after the dose, and before the next scheduled dose. Food intake was maintained unchanged on days 1 and 2. The protocol was prolonged for 72 h in the adult. We had the opportunity to collect the expired gas twice in the same 19-month-old infant (patient L) at different sampling times (before and at 2, 4, and 6 h after dose administration). The collection was performed first with the infant breathing quietly and 1 min later with the infant crying during the whole gas collection.

Small excesses of exogenous ^{13}C-CO_2 arising from ^{13}C-tri CAF demethylation were assessed by isotope ratio mass spectrometry. CO_2 was cryogenically isolated from respiratory gases using a −94 °C frozen methanol slush bath to trap water and a −196° C liquid nitrogen bath to trap CO_2 (7). The ^{13}C enrichment of expired CO_2 was calculated from the ratio of the intensities of the peaks ^{12}C-CO_2 + (m/z = 44) and ^{13}C-CO_2 + (m/z = 45) measured with a dual inlet isotope ratio mass spectrometer (modified ATLAS CH4). The ^{13}C enrichments were expressed as the per mil difference from the $^{13}C/^{12}C$ ratio of a CO_2 standard (8). Analysis of several aliquots (n = 10) of the same sample indicated a precision (1 SD) of isotopic analysis of 0.4/mil. The excess of exogenous ^{13}C-CO_2 was calculated from the increase of the per mil ^{13}C baseline. The per mil ^{13}C baseline was determined from the average of the values obtained for endogenous ^{13}C-CO_2 on day 1.

The concentration of CO_2 in the breath samples was determined by isotope dilution mass spectrometry using ^{13}C-CO_2 as internal standard. A known amount of ^{13}C-CO_2 was added to an exact amount of breath sample. After equilibration, CO_2 was cryogenically isolated and the ^{13}C enrichment was used to calculate the concentration of CO_2 in the breath samples. The CO_2 production rate was calculated by multiplying the CO_2 concentration in the breath sample by the respiratory flow rate (see Appendix 1).

The instantaneous labeled CO_2 excretion was calculated as percent of ^{13}C-tri CAF administered per mM of expired CO_2 according to Schneider (9). The instantaneous labeled CO_2 excretion rate as percent of ^{13}C-tri CAF administered was obtained by multiplying the percent of administered ^{13}C-tri CAF/mol of CO_2 by the CO_2 excretion rate. The cumulative labeled CO_2 excretion as percent of ^{13}C-tri CAF administered for a given period of time was obtained from the area under the curve of the instantaneous labeled CO_2 excretion rate as a function of time.

Blood samples of 500 μl were drawn by heelprick immediately after each breath sample collection on day 2. Blood samples were centrifuged and plasma was kept at +4° C for less than 24 h. Each urine sample was collected separately between two consecutive doses on day 2 and frozen at −20° C. The plasma concentration of CAF and metabolite concentrations in urines were determined using high-performance liquid chromatography as described previously (5, 10). Metabolite concentrations in urine were determined on pooled complete consecutive collections.

The CAF pharmacokinetic parameters were calculated using a one-compartment model (5). The CAF total clearance was calculated as the ratio of the administered dose, assuming 100% bioavailability, to the area under the time-CAF plasma concentration curve between two consecutive doses. CAF demethylation was estimated from the metabolite elimination in urine as previously described (10). The percentage of methyl groups eliminated from CAF was calculated from the ratio of the number of methyl groups absent in the metabolites recovered in urine to the number of methyl groups contained in the molecules of the parent CAF (Appendix 2).

Kinetic and statistical computations were performed with a Tektronix (4050) micro computer using our own software (TRIOMPHE). Each patient was counted only once in the calculations unless otherwise stated. The results are expressed as mean and SD. The multiple linear regression analysis was used to test the influence of postnatal age and gestational age. The linear regression analysis was used to test the correlation between ^{13}C-CO_2 excretion and CAF plasma clearance and between ^{13}C-CO_2 excretion in a patient breathing quietly and ^{13}C-CO_2 excretion in the same patient while crying. The linear regression analysis was also used to test the influence of postnatal age and, when necessary, the nonlinear regression based on a Gauss Newton-type algorithm was used.

RESULTS

The CAF breath test plasma clearance and urinary metabolite data are shown in Table 2. Multiple regression analysis did not reveal any linear correlation of the ^{13} C-tri CAF demethylation expressed as percent cumulative dose 2, 4, and 6 h after dose administration with postnatal or gestational ages. However, no detectable changes in exhaled ^{13}C-CO_2 from basal levels were observed in neonates and young infants whereas changes were measurable in all infants older than 33 days of age and 44 wk postconceptional age. These data imply that there is some influence of maturation on CAF demethylation. The changes in 2 h cumulative excretion of ^{13}C-tri CAF as ^{13}C-CO_2 as a function of postnatal age are presented in Figure 1*A*. A similar trend was observed for the variations of the 4- and 6-h cumulative ^{13}C-tri CAF excretion (data not shown). The values obtained in the two patients in whom sequential studies were performed at different postnatal ages are consistent with those obtained in the whole population. Those obtained in the adult patient are within the range of published values for nonsmoking healthy adults (11).

Using multiple regression analysis we found a significant influence ($p < 0.01$) of postnatal age on CAF plasma clearance, but in contrast to our earlier study (5), we found no influence of gestational age. The variation of CAF plasma clearance with postnatal age is shown in Figure 1*B*. The increase of CAF plasma clearance with postnatal age fits better with a single exponential curve (r = 0.914) than with a straight line (r = 0.786). The plateau (0.118 liter/kg/h) is reached at approximately the age of 6 months. In patients G and H the changes in CAF plasma clearance at different postnatal ages are consistent with those found in the other patients. The value found in the adult (0.0680 liter/kg/h) is within the range of those reported in nonsmoking healthy adults (12).

The urinary CAF metabolite data are presented in Figure 1*C*.

Table 2. *Results for CAF breath test, plasma clearance, and urinary metabolites*

Patient	Dose ^{13}C-tri CAF (mg base/kg)	Daily dose CAF (mg base/kg)	^{13}C-CO_2 (cumulative % dose) 2 h	4 h	6 h	Plasma clearance (l/kg/h)	Urinary metabolites demethylation
A	2.43	2.43	0.0	0.0	0.0	0.0097	0.331
B	2.50	2.50	0.0	0.0	0.0	0.0067	0.114
C	2.57	2.57	0.0	0.0	0.0	0.0072	0.457
D	2.36	2.36	0.0	0.0	0.0	0.0068	0.194
E	2.34	2.34	0.0	0.0	0.0	0.0129	0.491
F	5.29	5.29	0.0	0.0	0.0	0.0223	0.279
G(I)	3.55	7.10	1.6	4.4	6.2	0.0368	0.560
H(I)	2.59	2.59	0.3	2.0	4.2	0.0293	0.516
I	9.86	19.72	4.4	14.1	22.8	0.1533	0.544
J	4.71	14.13	3.0	8.8	14.7	0.1022	0.592
K	6.18	24.72	0.6	2.1	4.0	0.0758	0.583
L	6.59	19.77	1.4	3.7	5.8	0.1436	0.583
G(II)	4.76	9.52	0.3	1.5	4.2	0.0679	0.612
H(II)	2.72	5.44	3.2	8.1	12.2	0.0359	0.562
H(III)	4.41	13.23	1.2	3.9	7.4	0.0619	0.637
H(IV)	6.42	19.26	1.3	4.7	8.8	0.0752	0.553
Adult	4		1.3	2.54	4.17	0.0680	0.536

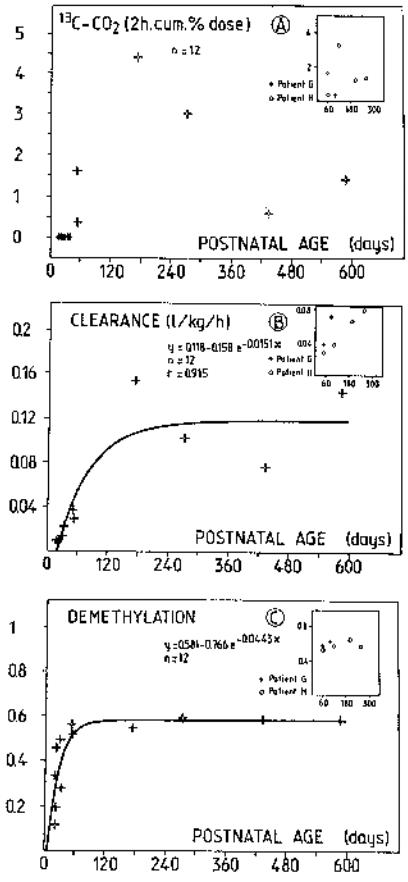

Fig. 1. *A*, changes in ^{13}C-tri CAF demethylation as exhaled ^{13}C-CO_2 (percent cumulative dose 2 h after ^{13}C-tri CAF administration) with postnatal age in 12 infants. *B*, changes in CAF plasma clearance with postnatal age in the same 12 infants. *C*, changes in CAF demethylation ratio as urinary metabolites with postnatal age. The percentage of methyl groups eliminated from caffeine was calculated from the ratio of the number of methyl groups absent in the metabolites recovered in urine to the number of methyl groups contained in the molecules of the parent CAF. In the *inset* patient G and H were studied serially.

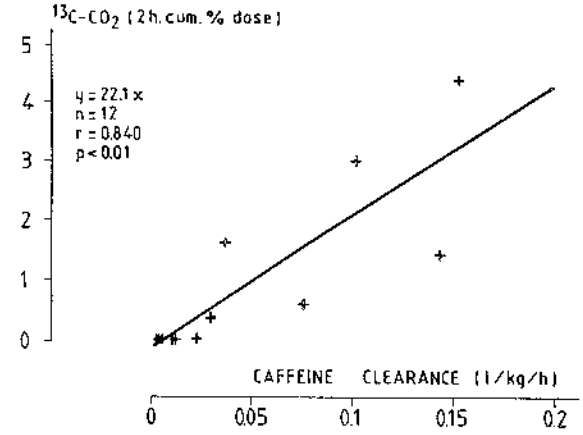

Fig. 2. Correlation between the 2-h cumulative excretion of ^{13}C-tri CAF as exhaled ^{13}C-CO_2 and CAF plasma clearance.

The percentage of all CAF methyl groups metabolized increases with postnatal age and this relationship is well described by a monoexponential curve. The plateau is reached by the age of 120 days and accounts for about 60% of all CAF methyl groups. In the two patients the values are consistent with those found in the whole population. The value found in the adult patient (53.6%) is within the range of those we reported in healthy adults (56.3 ± 4.99%) (10).

A significant correlation was obtained between the 2-h cumulative excretion of ^{13}C-tri CAF as ^{13}C-CO_2 and CAF plasma clearance as shown in our 12 patient group (Fig. 2) ($r = 0.840$). The correlations obtained for the 4-h ($r = 0.828$) and 6-h ($r = 0.838$) cumulative excretion were similar (data not shown).

Data on the effect of crying on breath CO_2 in one infant are presented in Table 3. We found a good correlation between the different cumulative doses calculated with the patient breathing quietly and those calculated during crying ($r = 0.999$, $p < 0.01$). We found that the values obtained with the patient crying were on the average 64.7% of those obtained while breathing quietly. Nevertheless the values of delta per mil obtained while crying were equivalent to those obtained while breathing quietly as demonstrated by the linear regression [$r = 0.993$, $p < 0.001$, slope = 1 (data not shown)]. This would suggest that the differ-

Table 3. *CAF breath test in patient L breathing quietly (Q) vs crying (C)*

Time (h)	Δ/‰		% ^{12}C-CO_2 in expired gas		Rate of expired gas (ml/mn)		^{12}C-CO_2 excretion rate (mmol/kg/h)		^{13}C-CO_2 cumulative % dose	
	Q	C	Q	C	Q	C	Q	C	Q	C
0	10.0	10.4	2.7	3.2	2334	1812	18.8	17.1	0.00	0.00
2	18.1	16.8	2.9	3.2	3064	2110	26.2	19.7	2.20	1.40
4	16.8	16.6	2.4	2.5	2893	1712	20.8	12.9	5.95	3.70
6	17.2	16.6	1.9	3.3	3219	1898	17.7	18.4	8.80	5.80

ence between the quiet and crying condition is mostly related to differences in unlabeled CO_2 excretion rate. Although we did not find any correlation between the values of CO_2 excretion rate in the two situations (not shown), there is a tendency for the CO_2 excretion rate as well as the expired flow rate to be lower in the crying condition compared to the quiet condition, whereas the percent CO_2 in the expired gas seems to be higher in the crying condition.

DISCUSSION

In humans as well as in rats, CAF is primarily metabolized via N-demethylation (13, 14). The N-demethylation of CAF and CAF metabolites produces formaldehyde which is further oxidized to CO_2 (15). Several experimental results in animals strongly suggest that CAF N-demethylation is dependent on P450-dependent isoenzymes induced by 3-methylcholanthrene but not by phenobarbital (16–18); they are also called P_1450 or P448 and now P450I according to the recently recommended nomenclature (19). Other experiments suggest that the labeled CO_2 exhaled in the breath during the CAF CO_2 breath test in animals correlates with the activity, measured *in vitro*, of P450I-dependent isoenzymes as well as hepatic cytochrome P450I concentration (20, 21). In man, it has been demonstrated that the rate of excretion of CO_2 derived from the N-demethylation of labeled ^{13}C-tri CAF is increased by cigarette smoking, probably because of exposure to polycyclic aromatic hydrocarbons present in the smoke. This suggests that CAF N-demethylation is dependent on a cytochrome P450I-dependent enzyme (11, 19). The CAF CO_2 breath test that monitors the N-demethylation of labeled CAF is therefore considered as a measure of the activity of P450I isoenzymes.

In man it is not ethically possible to perform studies comparing the CO_2 breath test with *in vitro* specific enzyme activity. Therefore only indirect methods of validating the CO_2 breath test as a monitor of P450I activity have been used. The plasma CAF clearance is used in adults assuming that labeled CO_2 excretion rate, P450I-dependent CAF N-demethylation, and CAF clearance correlate with one another. This assumption should be valid although CAF is metabolized by different pathways; N-demethylation is a major route of CAF metabolism in man (13). Furthermore CAF is a drug with a low hepatic extraction ratio (22) and a low plasma protein binding (23). CAF clearance is therefore not expected to be sensitive to changes in either hepatic blood flow or plasma protein binding but rather to intrinsic clearance which depends on the activity of the enzymes involved in CAF metabolism. The CAF CO_2 breath test has been shown to correlate with CAF plasma clearance in healthy smoking and nonsmoking adults and in patients with hepatic dysfunction (11, 24). We also found such a correlation in young infants. Despite the small number of gas samples collected in each patient, the correlation was about as strong as those reported in adults in the literature [$r = 0.90$ (11); $r = 0.83$ (24)].

Using the aminopyrine breath test that monitors the phenobarbital-inducible isoenzymes of P450, age-related changes in aminopyrine demethylase activity have been demonstrated in rats (25), in 1- to 38-wk-old infants (26) and in older children (27). The maturation of activity measured *in vitro* correlates with drug elimination *in vivo* (25). Some infants 14 to 19 wk of age had rates of elimination similar to those measured in adults (26). These data suggest that the P450 system undergoes maturational changes in activity (28).

Using the CAF breath test that monitors the P450I isoenzymes, Lambert *et al.* (29) showed age-related changes in demethylase activity in children older than 3 yr old. To our knowledge results in younger infants have never been published. The results of our study suggest that the capacity to eliminate the CAF-labeled methyl groups increases with age. The increase in elimination of the label more likely reflects the maturation of P450I-dependent CAF N-demethylation. The findings of this investigation are consistent with the increases of both the serum clearance of CAF (5) and the percentage of removed methyl groups of CAF in the urine metabolites with age (10). In newborn and young infants CAF clearance is very low and the exhaled ^{13}C-CO_2 in the breath test does not change from basal levels. In the older infants CAF clearance is high and even higher than in adults and the change in the exhaled ^{13}C-CO_2 in the breath test is easily measured and somewhat higher than in adults. However, some demethylation activity was demonstrated by the analysis of metabolites in urine as early as 33 wk gestational age and 35 wk postconceptional age whereas no increase in ^{13}C-CO_2 from basal levels was detectable after labeled CAF administration. Although the clearance of drugs with low extraction ratio is most sensitive to changes in intrinsic clearance, the usefulness of labeled CAF as a marker for N-demethylase activity is offset by the limit of detection of the method of analysis for ^{13}C-CO_2 in breath, using small CAF maintenance doses in young infants.

Expression of the results as the 2-h cumulative excretion of CO_2 provided the highest correlation between the labeled CO_2 excretion and CAF clearance in human adults (11, 24). Cumulative labeled CO_2 excretion at later times become more dependent on CAF metabolite demethylation. However, the 2-h cumulative labeled CO_2 excretion appears to be less sensitive to dose to dose variability in the absorption rate than those based on 30-min and 1-h excretion rates (30), especially if it is obtained from the area under the excretion rate-time curve of multiple samples. Inasmuch as no gas collection was performed between 0 and 2 h after ^{13}C-tri CAF administration in our study, the 2-h cumulative excretion of ^{13}C-tri CAF as ^{13}C-CO_2 was calculated from the area under the instantaneous labeled CO_2 excretion rate-time curve using only the 2 h instantaneous excretion point. It was therefore calculated from the triangular integration of the curve between 0 and 2 h and equal to the 2-h instantaneous excretion of ^{13}C-tri CAF as ^{13}C-CO_2. The correlations between cumulative excretion of ^{13}C-tri CAF as ^{13}C-CO_2 and CAF clearance were not stronger at 4 or 6 h than at 2 h, suggesting that a single instantaneous gas collection 2 h after labeled CAF administration would be sufficient to estimate CAF clearance in future studies.

Methyl groups arising from CAF N-demethylation appear in the breath as CO_2 only after several further oxidation steps with formaldehyde, formic acid, and carbonic acid as intermediates. The label has to pass through several intermediary metabolite pools resulting in dilution of the label and losses via other routes

of metabolism. The rate of ^{13}C-CO_2 formation therefore reflects the rate of N-demethylation of the administered 13-tri CAF only if the modifications occuring at the level of the intermediary metabolism are predictable, *i.e.* remain the same for all the experimental conditions.

During gas collection the young infants cry because the face mask is firmly secured to avoid leaks and guarantee an accurate collection of expired air to estimate the rate of CO_2 production. Therefore the respiratory pattern cannot be even, and the effect of crying may contribute to inter- and intraindividual variability in CO_2 production. It has been shown in babies that the face mask itself results in a significant fall in respiratory frequency and an increase in tidal volume, and the use of both mask and pneumotachograph produces significant increase in minute ventilation (31, 32). Although not statistically significant, the results obtained in our patients for CO_2 excretion rate and expired flow rate suggest underbreathing while crying. If the patient underbreathes, he or she will expire less CO_2 than the cells are producing during the period of underbreathing. This results in a falsely low estimate of the rate of CO_2 production: the assumption of steady state in pool sizes is not verified. In one of our patients we had the opportunity to show the influence of crying on the labeled CO_2 excretion rate. The cumulative labeled CO_2 excretion was consistently lower while crying and on the average was equal to 65% of the values obtained when the same patient was breathing quietly. Thus the ^{13}C-CO_2 excretion rate was underestimated during the crying state. To identify the difference of the labeled CO_2 excretion related only to labeled CAF N-demethylation and to minimize the variability related to crying in the labeled CO_2 excretion rate, gas collections were performed the day before labeled CAF administration according to the same protocol as on the day after this administration.

Despite these difficulties our study shows that the CAF CO_2 breath test is feasible in infants. It is a safe and noninvasive method useful to determine age-related changes in P_1450-dependent N-demethylase activity and to correlate the labeled CO_2 excretion rate to CAF plasma clearance. However, because of this important variability the value of the use of this correlation for quantitative measurement of CAF clearance and monitoring has to be evaluated.

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APPENDIX 1

$$\delta\ ‰\ ^{13}C = \frac{[(^{13}C/^{12}C \text{ sample} - (^{13}C/^{12}C) \text{ standard})]}{(^{13}C/^{12}C) \text{ standard}} \times 10^3$$

$$\frac{\%\ \text{administered}\ ^{13}\text{C-tri-CAF}}{\text{mmol expired total } CO_2} = \frac{\Delta\ ‰\ ^{13}C \times M \times (^{13}C/^{12}C) \text{ standard}}{10 \times P \times n \times m}$$

where $\Delta\ ‰\ ^{13}C = (\delta\ ‰\ ^{13}C)$ sample $- (\delta\ ‰\ ^{13}C)$ baseline; M, mol. wt. of labeled compound (=197); n = number of labeled atoms per molecule (=3); P = ^{13}C isotopic enrichment of labeled compound (=0.98); and m = mg of ^{13}C-tri-CAF administered.

APPENDIX 2

Ratio used for the estimation of the demethylation process (ratio of the number of methyl groups absent in the metabolites recovered in urines to the number of methyl groups contained in the molecules of the parent CAF).

$$\frac{[(DX + DU) \times 1] + [(MX + MU) \times 2]}{\text{Total} \times 3}$$

$$\text{Total} = (DX + DU + MX + MU + AFMU + TX + TU)$$

Where TX = trimethylxanthine (CAF); DX = dimethylxanthines; MX = monomethylxanthines; DU = dimethyluric acids; MU = monomethyluric acids; TU = uric acid; AFMU = 5 acetylamino 6 formyl-amino 3 methyluracil. The values for CAF and metabolites are expressed as mol.