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Determination of human ketone body kinetics using stable-isotope labelled tracers

M. Beylot[1], B. Beaufrère[1], S. Normand[1], J. P. Riou[1], R. Cohen[2] and R. Mornex[1]

[1]INSERM U. 197, Faculté de Médecine Alexis Carrel, Lyon, and
[2]Service of Radioanalyse, Hôpital Neurocardiologique, Bron, France

Summary. In order to avoid the use of radioactive tracers for the determination of human ketone body turnover, we have developed a method using a primed-continuous infusion of ^{13}C-labelled acetoacetate or D-β-hydroxybutyrate. Determination of the mole percent enrichment of blood acetoacetate and D-β-hydroxybutyrate was performed by gas chromatography/mass spectrometry. In the post-absorptive state, the mean total ketone body appearance rate, determined in four subjects, was 3.74 μmol·kg^{-1}·min^{-1} using [3,4-^{13}C2] acetoacetate and 2.76 μmol·kg^{-1}·min^{-1} using [3-^{13}C]D-β-hydroxybutyrate, values in agreement with those reported in studies with ^{14}C-labelled tracers. In order to evaluate the usefulness of the method for determination of ketone body kinetics in non steady-state conditions, we infused four subjects with natural sodium acetoacetate and calculated the isotopically determined total ketone body appearance rate using a single compartment model (volume of distribution 0.20 l/kg; functional pool fraction: 1). During the tests with [3,4-^{13}C2]-acetoacetate, the actual infusion rates of natural acetoacetate were 7.3 ± 0.3, 14.6 ± 0.8, 21.9 ± 1.2 and 10.9 ± 0.6 μmol·kg^{-1}·min^{-1} whereas the corresponding isotopically determined total ketone body appearance rates were respectively 9.2 ± 1.0, 16.3 ± 0.7, 23.1 ± 1.1 and 10.7 ± 0.8 μmol·kg^{-1}·min^{-1}. During the tests with [3-^{13}C]D-β-hydroxybutyrate, the actual infusion rates were 8.4 ± 0.5, 16.8 ± 0.9, 25.2 ± 1.4 and 12.6 ± 0.8 μmol·kg^{-1}·min^{-1}, and the isotopically determined appearance rates respectively 11.1 ± 0.7, 16.7 ± 0.7, 25.0 ± 1.1 and 11.1 ± 0.7 μmol·kg^{-1}·min^{-1}. Thus, using either tracer we found a good agreement between acetoacetate infusion rate and tracer-determined appearance rate of ketone bodies. In conclusion, the present method may be used to determine human ketone body kinetics under steady-state and non steady-state conditions.

Key words: Acetoacetate, β-hydroxybutyrate, ketogenesis, ketone body kinetics, stable isotopes, mass spectrometry.

Blood ketone bodies (acetoacetate and D-β-hydroxybutyrate) are important metabolic substrates, since they can act as oxidative fuels, as lipogenic precursors and as regulators of metabolism [1]. An understanding of their in vivo metabolism requires the determination of their appearance and disappearance rates. Such determinations usually are performed using the isotope dilution method with ^{14}C-labelled acetoacetate or D-β-hydroxybutyrate [2-7]; these methods have been validated for both steady- and non steady-state conditions [4, 5, 8]. However, radioactive tracers expose patients to the hazards of radiation, which makes their use undesirable in adults and precludes their utilisation in children or pregnant women. Moreover, the use of radioactive isotopic tracers in normal subjects is not authorized in some countries. The use of stable isotopes avoids these limitations. Recently, Miles et al. [9] have reported a method for the determination of ketone body turnover in dogs using the γ-butyldimethylsilyl derivatives of ketone bodies and stable-isotope labelled tracers. In the present report we describe a method using trimethylsilyl derivatives of ketone bodies and gas-chromatography/mass spectrometry (GC/MS) for determination of the enrichment of blood acetoacetate and D-β-hydroxybutyrate during infusion of ^{13}C-labelled tracers, and the application of the method for the determination of ketone body flux under both steady- and non steady-state conditions in humans.

Subjects and methods

Four normal subjects [3 men and 1 woman aged 29 to 40 years (body mass index 20.2 to 24.8)] volunteered for the study after full explanation of the purpose, nature and possible risks of the investigations. All these in vivo studies were performed in accordance with the principles of the Declaration of Helsinki. The subjects were instructed to consume their usual diet throughout the study. All tests were performed in the post-absorptive state. Each subject had two tests, one

with [3-^{13}C]D-β-hydroxybutyrate and the other with [3,4-^{13}C2]-acetoacetate, with an interval of at least one week between tests. On the morning of the test an indwelling catheter was placed into an antecubital vein for the infusions; to obtain arterialized blood samples another catheter was inserted in a retrograde manner in a vein of the opposite hand kept at 60 °C in a warming box [10]. Patency of all catheters was maintained by a slow infusion of isotonic saline. After 30 min of bed rest, three blood samples were collected at 5 min intervals. A priming dose (10 times the infusion rate over 1 min) of the tracer was then injected followed by a constant infusion over 165 min ([3-^{13}C]D-β-hydroxybutyrate: 0.08–0.12 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ or [3,4-^{13}C2] acetoacetate 0.144–0.223 $\mu mol \cdot kg^{-1} \cdot min^{-1}$). Tracers were diluted with sodium chloride (154 mmol/l) prior to infusion with a previously calibrated pump (Bioblock Scientific, Strasbourg, France). Following a basal equilibrating period of 90 min, natural sodium acetoacetate was infused with a peristaltic pump (Dubernard Hospital, Bordeaux, France) at four successive rates (8, 16, 24 and 12 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, each for 15 min. Blood samples were collected before the infusion of natural sodium acetoacetate (60–90 min), at each infusion step, and 10 and 15 min afterwards. Aliquots of the tracers were collected before and after the infusion in order to check concentration and stability. No spontaneous degradation of either tracer was observed (data not shown). The infusion rates of natural acetoacetate were determined from the concentration and volume of acetoacetate delivered during each period of infusion.

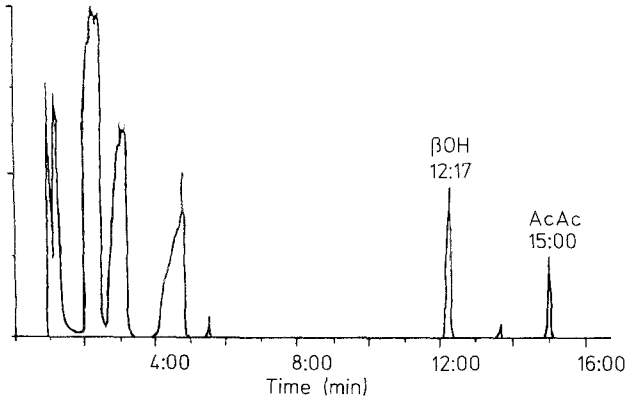

Fig. 1. Total ion chromatogram obtained with the trimethylsilyl derivatives of a mixed standard of acetoacetate and D-β-hydroxybutyrate. The peaks observed during the first 5 min are solvents and reagents. D-β-hydroxybutyrate eluates next, followed by the two peaks of acetoacetate

Preparation of infusates

[3-^{13}C] ethyl acetoacetate (99% atom excess) and [3,4-^{13}C2]ethyl acetoacetate (99% atom excess) were obtained from the "Commissariat à l'Energie Atomique" (Gif-sur-Yvette, France). For the preparation of labelled acetoacetate [3,4-^{13}C2]ethyl-acetoacetate was hydrolysed by incubation at 40 °C during 60 min with 10% excess of 1 N NaOH. After neutralisation with 1 N HCl, the solution was washed three times with diethylether (Merck, Darmstadt, FRG), flushed with N_2 and stored at −80 °C until use. Natural sodium acetoacetate was prepared from ethyl-acetoacetate (Merck) by the same procedure, adjusting the pH to 7.40 prior to storage at −80 °C. (Yield 90–99%).

For the preparation of labelled D-β-hydroxybutyrate, [3-^{13}C]ethyl acetoacetate was first hydrolysed to acetoacetate as described. [3-^{13}C]acetoacetate was then converted to [3-^{13}C]D-β-hydroxybutyrate using β-hydroxybutyrate dehydrogenase (Boehringer, Mannheim, FRG) and NADH (Boehringer) in phosphate buffer (10 mmol/l, pH 6.80). The reaction was followed by measuring the decrease in optical density at 340 nm. (Yield >90% after 48 h at room temperature). β-hydroxybutyrate dehydrogenase was removed by ultrafiltration (PM 10, Amicon Corporation, Lexington, MA, USA) and the filtrate subjected to anion exchange chromatography (AG1X8 in the HCO_3^- form, Biorad, Richmond, CA, USA). NAD, acetoacetate and D-β-hydroxybutyrate were eluted with $NaHCO_3$ (10 g/l), NADH remaining on the column. The eluate was concentrated and acidified to pH 1 with HCL and D-β-hydroxybutyrate extracted with diethylether. Finally, the organic phase was evaporated in a vial containing $NaHCO_3$ (20 ml, 10 g/l). The purified sample contained no detectable trace of NAD or NADH. The small amount of acetoacetate remaining with D-β-hydroxybutyrate was destroyed by heating at 100 °C for 1 h and flushing with N_2. (Overall yield 75–80%). All infusates were passed through a 0.22 µm Millipore filter following preparation. The absence of pyrogen was checked by the standard method of measurement of rabbit body temperature after intravenous injection.

Analytical procedures

Acetoacetate, D-β-hydroxybutyrate and glucose were assayed in whole blood after deproteinization by 6% ice cold perchloric acid using standard enzymatic methods as described previously [11]. Ace-

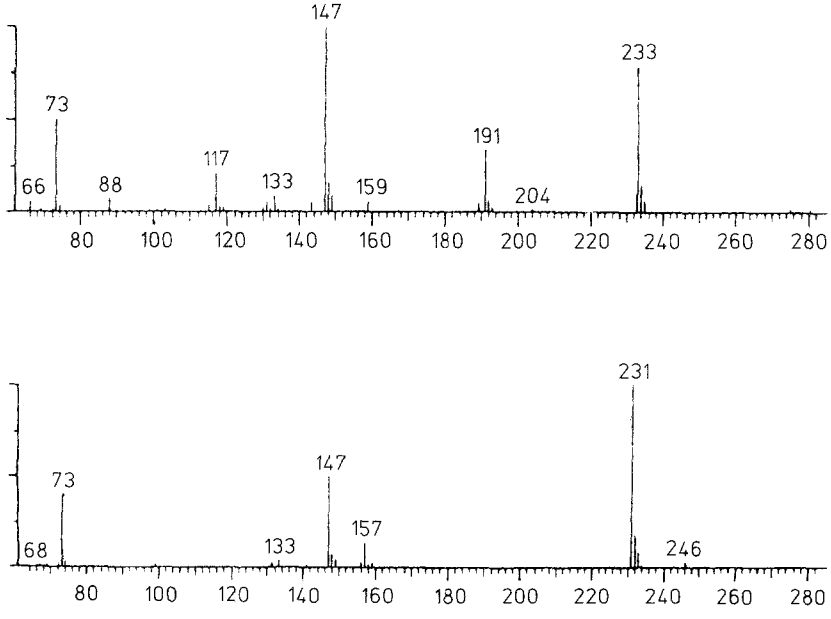

Fig. 2. Mass spectra of the trimethylsilyl derivatives of acetoacetate (lower part) and D-β-hydroxybutyrate (upper part). The ions of m/z 231 for acetoacetate and 233 for β-hydroxybutyrate represent the molecular ions less a methyl group and were used for the determination of ketone body enrichment

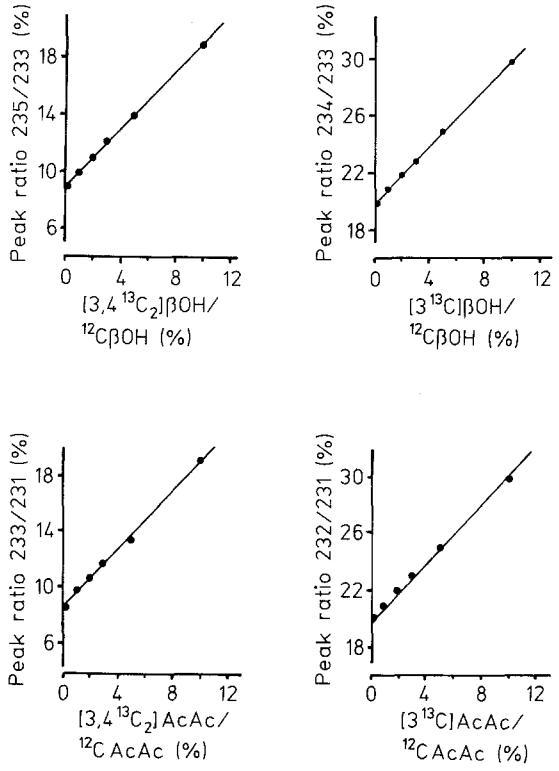

Fig. 3. Standard curves used for the determination from peak ratios of the ratios of labelled (^{13}C) over natural (^{12}C) acetoacetate (lower part) and D-β-hydroxybutyrate (upper part) using either [3,4-^{13}C2]-labelled tracer (left part) or [3-^{13}C]-labelled tracer (right part). $r=0.999$ for each standard curve. Due to the natural abundance of stable isotopes in the ditrimethylsilyl derivatives of ketone bodies (mainly $^{29}S1$, $^{30}S1$ and ^{13}C) the intercepts (peak ratios for a labelled over natural ketone bodies ratio of zero) of the standard curves are 8.86 (235/233) and 8.76 (233/231) when using [3,4-^{13}C2] labelled tracer (theoretical value 8.89). When using [3-^{13}C] labelled tracer the intercepts are 19.85 (234/233) and 19.96 (232/231) (theoretical value: 19.90)

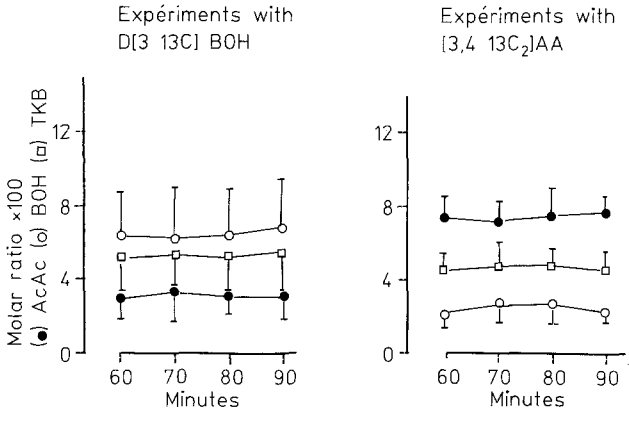

Fig. 4. Molar ratios observed in acetoacetate (●——●), β-hydroxybutyrate (○——○) and total ketone bodies (□——□) following infusion with the labelled tracers D[3-^{13}C] β-hydroxybutyrate, [3,4-^{13}C2] acetoacetate in the post-absorptive state. Infusion of the tracer was started at time 0. Stable levels were obtained from 60 to 90 min

toacetate was assayed within 8 h. Immunoreactive insulin (IRI) and glucagon (IRG) were determined [12, 13] on plasma kept frozen until the day of the assay. Blood for glucagon determination was collected in tubes containing EDTA and trasylol. Plasma non-esterified fatty acids (NEFA) were determined enzymatically [14].

For determination of the isotopic enrichment of acetoacetate and D-β-hydroxybutyrate, 2 ml of blood were collected on ice cold perchloric acid (6%) and centrifuged immediately. The neutralized supernatant was stored frozen until analysis. Two procedures were used. For most samples, 1 ml aliquots were acidified to pH 1 with HCL, mixed with 9 ml of ethyl acetate (Prolabo, Paris, France) and shaken vigorously for 60 s. After separation, the organic phase was evaporated to dryness under a stream of N_2 at room temperature prior to derivatization. For some samples, 1 ml of sample was placed on an anion exchange resin column (AG1X8 formate form, Biorad, Richmond, CA, USA), the resin washed with deionized water (3 ml) and acetoacetate and β-hydroxybutyrate eluted with perchloric acid (1 ml). After neutralization and centrifugation the eluate was lyophilized before derivatization. Similar results were obtained with both methods. Derivatives were synthesised by adding pyridine (20 µl, Pierce Chemical Company, Rockford, IL, USA) and bis(trimethylsilyl)trifluoracetamide (20 µl) with 1% of trimethylchlorosilane (Pierce Chemical Company) at room temperature. Aliquots (1 µl) were injected into a gas chromatograph (Fractovap 4160, Carlo Erba) coupled with a mass spectrometer (Quadrupole R 10-10, Nermag, Rueil Malmaison, France) and equipped with a 10 m fused silica capillary column coated with CP Sil 19 CB (Chrompack Inc., Bridgewater, NJ, USA).

The operating conditions were as follows: injector temperature 170 °C, oven temperature 50 °C for 4 min increasing by 5°/min; split ratio 1/10, interface temperature 180–200 °C, source temperature 200 °C. Fragmentation of the molecule was performed by electron impact (70 ev) and the following ions were selectively traced: m/z 231, 232, 233 for acetoacetate and 233, 234, 235 for D-β-hydroxybutyrate.

Calculations

The peak area ratios (m/z 232/231 and 233/231 for acetoacetate and m/z 234/233 and 235/233 for β-hydroxybutyrate) were calculated and the corresponding molar ratios were determined from standard curves prepared by mixing weighed amounts of natural and ^{13}C-labelled acetoacetate and β-hydroxybutyrate (see Results) before calculation of the moles percent enrichment (MPE) of blood acetoacetate and D-β-hydroxybutyrate [MPE ≡ tracer/(tracer + tracee)]. At steady-state the appearance rates (µmol · kg^{-1} · min^{-1}) of total ketone bodies (TKB) were calculated by the formula:

$$\text{TKB Ra} = \frac{F}{\dfrac{\text{MPE A [A]} + \text{MPE B [B]}}{\text{[A]} + \text{[B]}}} - F$$

where F is the isotope infusion rate (µmol · kg^{-1} · min^{-1}), [A] and [B] are the concentrations of acetoacetate and β-hydroxybutyrate (µmol/l), and MPE A and MPE B are the moles percent enrichments of acetoacetate and D-β-hydroxybutyrate.

In this formula the term $\dfrac{\text{MPE A [A]} + \text{MPE B [B]}}{\text{[A]} + \text{[B]}}$ represents "total ketone body moles percent enrichment", an expression analogous to the "total ketone body specific activity" used for radioactive tracers [2–7].

During non steady-state conditions the appearance and disappearance rates of acetoacetate, D-β-hydroxybutyrate and total ketone bodies were calculated using the modified Steele's equations:

$$\text{Ra} = \frac{F - \dfrac{pV\,(C2+C1)}{2} \times \dfrac{(MPE2 - MPE1)}{t2 - t1}}{\dfrac{MPE2 + MPE1}{2}} - F$$

$$\text{Rd} = \text{Ra} - \frac{pV\,(C2 - C1)}{t2 - t1}$$

where V is the volume of distribution, p the functional pool fraction, t1 and t2 represent two different sampling points; C2 and C1 and MPE2 and MPE1 represent concentrations and MPE at t2 and t1 of

Table 1. Individual values in the post-absorptive state of the concentrations of ketone bodies, NEFA and of the appearance rate (Ra) of total ketone bodies (TKB) of the four subjects studied

Subjects	Tests with [3,4-$^{13}C_2$] acetoacetate				Tests with [3-^{13}C]D-β-hydroxybutyrate			
	Acetoacetate (μmol/l)	D-β-hydroxy-butyrate (μmol/l)	NEFA (μmol/l)	Ra TKB (μmol·kg^{-1}·min^{-1})	Acetoacetate (μmol/l)	D-β-hydroxy-butyrate (μmol/l)	NEFA (μmol/l)	Ra TKB (μmol·kg^{-1}·min^{-1})
1	96	280	419	4.50	75	164	310	2.45
2	26	50	290	2.52	80	123	380	2.49
3	65	108	386	3.50	40	102	235	2.76
4	72	150	520	4.45	32	45	296	2.18

acetoacetate, D-β-hydroxybutyrate or TKB as appropriate. The values of p and V used for calculations were 1 and 0.20 l/kg of body weight as previously established by Keller et al. [5].

Statistical analysis

Results are shown as individual values or mean and standard error of the mean. Correlations were established by the least square method. Comparisons were performed using Student's t-test for paired values.

Results

Gas chromatography-mass spectrometry procedures

A total ion chromatogram and the mass spectra of the trimethylsilyl derivatives of a standard of mixed acetoacetate and D-β-hydroxybutyrate are shown in Figures 1 and 2 respectively. The two peaks of acetoacetate have identical mass spectra (these two peaks represent probably the formation of tautomeric isomers due to the 2,3 double bond formation [9]). In preliminary experiments (standard curves and determinations of in vivo enrichment) the same results were obtained using either of these two peaks. The peak of greater abundance was therefore used. There was a small molecular ion for acetoacetate but not for β-hydroxybutyrate. The ions of m/z 231 for acetoacetate and 233 for β-hydroxybutyrate correspond to the molecular ion (m/z respectively 246 and 248) minus a methyl group (m/z 15). These ions were used for the determination of enrichment. During the tests with [3,4-^{13}C2] acetoacetate, the ions 231 and 233, and 233 and 235, were selectively monitored for acetoacetate and D-β-hydroxybutyrate respectively. For the tests with [3-^{13}C]D-β-hydroxybutyrate the ions monitored were 231 and 232 for acetoacetate and 233 and 234 for D-β-hydroxybutyrate. The natural enrichments of acetoacetate and β-hydroxybutyrate isolated from blood prior to infusion of tracer were identical to those obtained from unenriched standard. Accurate determinations of peak area ratios could be obtained for concentrations of ketone bodies as low as 15 μmol/l. Intraassay coefficients of variation were 1% or less for the peak ratios 232/231 and 234/233 and 1.5% for the ratios 233/231 and 235/233. Corresponding interassay coefficients of variation were respectively 3.8% and 5.1%. These coefficients of variation were determined using blood samples. Thereafter all blood samples of each test were assayed within the same series. Linear standard curves for acetoacetate and β-hydroxybutyrate were obtained using solutions of known enrichment (Fig. 3).

In vivo studies

During the infusion in the post-absorptive state of labelled acetoacetate or D-β-hydroxybutyrate, stable levels of concentration and enrichment were obtained for both ketone bodies from 60 min to 90 min (Fig. 4), thus permitting the use of formulae for steady-state conditions for determination of flux. However, as previously observed with ^{14}C labelled ketone bodies, there was an isotopic disequilibrium between acetoacetate and D-β-hydroxybutyrate. The calculated appearance rate of TKB during both experiments are shown in Table 1. The values of TKB appearance rates obtained during the tests with [3-^{13}C]D-β-hydroxybutyrate (2.76 ± 0.14 pmol·kg^{-1}·min^{-1}) were slightly lower than those found during the tests with labelled AcAc (3.74 ± 0.46 μmol·kg^{-1}·min^{-1}). However, plasma NEFA were also lower and there was a direct relationship between plasma NEFA concentration and TKB production rate ($r = 0.784$, $p < 0.05$).

Subsequent infusion of natural acetoacetate resulted in a decrease of NEFA levels (Table 2). The small decrease of blood glucose was not significant, and there were no detectable changes of immunoreactive insulin and glucagon concentrations.

During the experiments with [3,4-^{13}C2] acetoacetate (Fig. 5) the infusion of natural acetoacetate decreased the enrichment of both ketone bodies so that the enrichments of acetoacetate and D-β-hydroxybutyrate became almost identical. The actual infusion rates of acetoacetate were 7.3 ± 0.3, 14.5 ± 0.8, 21.9 ± 1.2 and 10.9 ± 0.6 μmol·kg^{-1}·min^{-1}, whereas the isotopically determined appearance rates were, at the end of each period of infusion, respectively 8.2 ± 0.7, 14.0 ± 0.3, 23.0 ± 1.7 and 9.8 ± 0.8 μmol·kg^{-1}·min^{-1} for acetoacetate and 9.2 ± 1.0, 16.3 ± 0.7, 23.1 ± 1.1 and 10.7 ± 0.8 μmol·kg^{-1}·min^{-1} for TKB. Thus, as previously found with ^{14}C labelled tracers [4], the maximal Ra values of acetoacetate and TKB were less than the sum of their basal values and the maximal rate of infusion of ace-

Table 2. Metabolite and hormone values before (basal period), during and after the infusion of natural acetoacetate

	Tests with [3,4$^{13}C_2$] acetoacetate						Tests with [3^{13}C]D-β-hydroxybutyrate					
	Basal period	During acetoacetate infusion				After infusion	Basal period	During acetoacetate infusion				After infusion
	60–90 min	105 min	120 min	135 min	150 min	165 min	60–90 min	105 min	120 min	135 min	150 min	165 min
Blood glucose (mmol/l)	4.3 ± 0.2	4.6 ± 0.2	4.3 ± 0.1	4.3 ± 0.2	4.0 ± 0.2	4.3 ± 0.1	4.6 ± 0.2	4.5 ± 0.3	4.4 ± 0.3	4.1 ± 0.2	4.3 ± 0.3	4.2 ± 0.1
AA (μmol/l)	64 ± 20	292 ± 45	500 ± 49	836 ± 68	530 ± 18	193 ± 30	60 ± 12	269 ± 46	508 ± 63	827 ± 25	514 ± 72	263 ± 21
BOH (μmol/l)	140 ± 55	206 ± 37	300 ± 32	489 ± 65	513 ± 65	265 ± 14	108 ± 20	199 ± 24	323 ± 28	571 ± 60	548 ± 88	387 ± 127
NEFA (μmol/l)	399 ± 60	292* ± 60	185* ± 25	134** ± 26	117** ± 21	171** ± 18	310 ± 25	265* ± 64	186* ± 39	147** ± 23	143** ± 26	130** ± 23
IRI (mU/l)	10.9 ± 0.5	10.8 ± 0.7	11.7 ± 1.2	12.7 ± 0.8	11.3 ± 0.4	9.5 ± 0.5	10.4 ± 0.6	10.8 ± 0.2	10.4 ± 0.6	10.4 ± 0.6	10.4 ± 0.6	8.9 ± 0.4
IRG (mg/l)	154 ± 50	160 ± 47	163 ± 64	171 ± 63	177 ± 51	158 ± 58	141 ± 52	160 ± 61	153 ± 49	147 ± 49	146 ± 51	119 ± 46

Results are shown as mean ± SEM, *, ** indicate a significant difference from values in the basal period at $p<0.05$ and <0.01 respectively

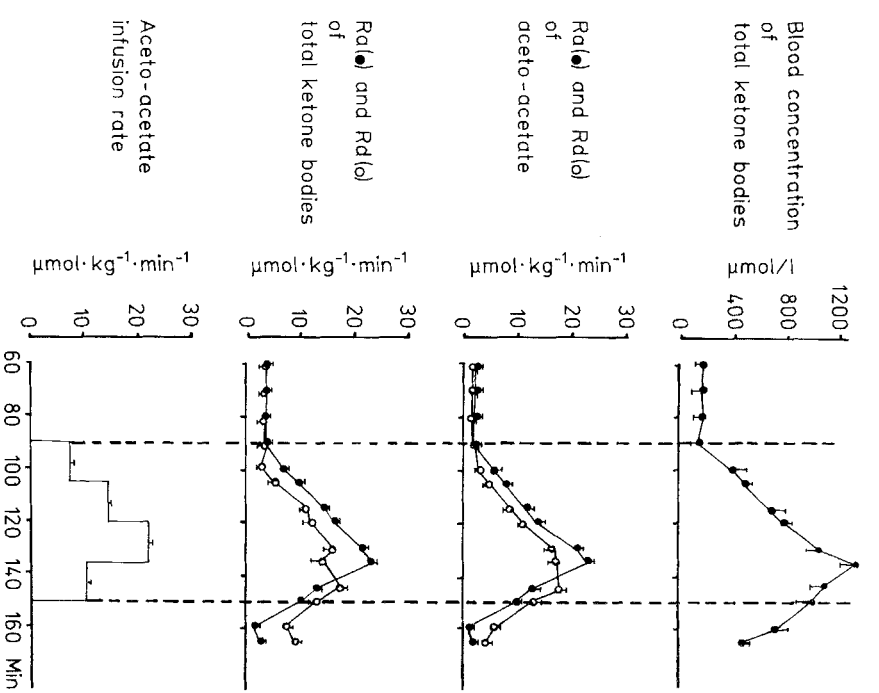

Fig. 5. Total ketone body (TKB) concentrations and the isotopically determined appearance rate (Ra, ●) and disappearance rate (Rd, ○) of acetoacetate and TKB in four subjects before and during the infusion of natural acetoacetate; experiments with [3,4-^{13}C2] acetoacetate

toacetate. Examination of the individual values showed a highly significant relation between the infusion rates of acetoacetate and the isotopically determined Ra of either acetoacetate ($r=0.94, p<0.001$) or TKB ($r=0.96$, $p<0.001$). Comparable results were obtained during the tests with [3-13C]D-β-hydroxybutyrate (Fig. 6). The infusion of natural acetoacetate also decreased the enrichment of both ketone bodies. The difference of enrichment between acetoacetate and D-β-hydroxybutyrate first increased slightly and then decreased progressively to near disappearance at the end of the test. The actual infusion rates of acetoacetate were successively 8.4 ± 0.5, 16.8 ± 0.9, 25.2 ± 1.4 and 12.6 ± 0.8 μmol·kg^{-1}·min^{-1}, whereas the isotopically determined Ra of TKB at the end of each infusion step were 11.1 ± 1.7, 16.7 ± 0.7, 25.0 ± 1.1 and 11.1 ± 0.7 μg·kg^{-1}·min^{-1}. Thus the maximal value of TKB Ra was again less than the sum of basal Ra value and maximum acetoacetate infusion rate. Examination of the individual values showed a highly significant relationship between acetoacetate infusion rates and isotopically determined Ra values of TKB ($r=0.96$, $p<0.001$). The production rate of D-β-hydroxybutyrate rose from a basal value of 1.95 ± 0.20 μg·kg^{-1}·min^{-1} to a maximal value of $11.6+0.6$ μg·kg^{-1}·min^{-1}, showing that a large part of

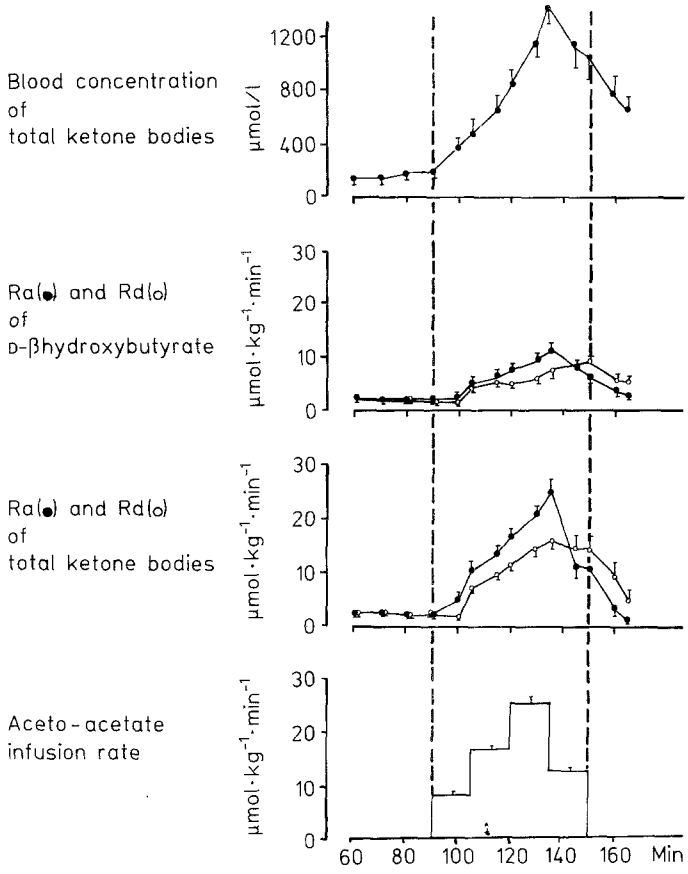

Fig. 6. Total ketone body (TKB) concentrations and the isotopically determined appearance rate (Ra, ●) and disappearance rate (Rd, ○) of D-β-hydroxybutyrate and TKB in four subjects before and during the infusion of natural acetoacetate. Experiments with [3-^{13}C]D-β-hydroxybutyrate

the infused acetoacetate was converted into D-β-hydroxybutyrate.

Discussion

In the present work we have developed a method for the determination of ketone body kinetics using stable-isotope labelled tracers and GC/MS systems. We have tested the usefulness of two different tracers, ^{13}C labelled acetoacetate and D-β-hydroxybutyrate, and found similar results using either compound. Labelled D-β-hydroxybutyrate has the advantage of stability, whereas acetoacetate is subject to spontaneous decarboxylation, but its preparation is tedious and time-consuming. Recently, Miles et al. [9] have published another method using stable isotopes for the determination of ketone body kinetics in dogs, but have not used it in man nor validated it for non steady-state conditions. Our method is relatively simple, not too time-consuming and allows determination of the enrichment even for the low ketone body levels of the post-absorptive state. The total innocuity of stable isotopes makes it possible to perform repetitive studies in adults and to conduct studies in pregnant women and children. The blood volume used, although relatively large, could be easily reduced for this last purpose.

It was impossible to compare our results with values obtained in the same subjects using ^{14}C labelled tracers, since the use of radioactive tracers for metabolic studies in normal subjects is not authorized in France. However, the TKB appearance rates in the post-absorptive state that we obtained in normal humans are consistent with previous results obtained using ^{14}C labelled acetoacetate or D-β-hydroxybutyrate. Miles et al. [15] report mean values of TKB production of 1.5 to 2.2 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, and Fery and Balasse [16] of 3.46 $\mu mol \cdot kg^{-1} \cdot min^{-1}$. Keller and Sonnenberg [4, 17] found slightly higher values (6 $\mu mol \cdot kg^{-1} \cdot min^{-1}$), but their subjects also had slightly higher TKB levels, and there is a direct relationship between TKB production and levels [18].

For determination of ketone body kinetics during the non steady-state, we have used a one pool model comparable to that used during experiments with ^{14}C labelled tracers [2-6]. The values of the volume of distribution and of the functional pool fraction used for the calculations were those previously established by Keller et al. [4] in normal humans. However, since these measurements were performed in post-absorptive normal subjects, use of this model in other situations would require prior determination of the effective distribution volume by the bolus injection technique. Using this single pool model we found a good agreement between the isotopically determined appearance rate of ketone bodies and the infusion rate of natural acetoacetate. Indeed, the calculated TKB appearance rates were slightly higher that the actual infusion rates of acetoacetate for the first infusion step in the experiments with labelled D-β-hydroxybutyrate and for the first and second infusion steps during tests with labelled acetoacetate. However, during the infusion of exogenous acetoacetate the TKB production is the sum of the endogenous production and of the infusion. There is evidence that endogenous ketone body production is decreased at least in part during infusion of ketone bodies [2, 4]. This possibility is supported, at least for the highest infusion rates, by the fall of NEFA levels observed in the present experiments as well as in previous studies [4, 19]. Moreover, although we failed to detect an increase of peripheral insulin concentration in the present studies, there is evidence that high ketone body levels can stimulate insulin secretion [19]. However, it is likely that some endogenous ketone body production persisted, especially during the initial infusion steps of natural acetoacetate; this could explain in part the small discrepancies between the isotopically determined appearance rates of TKB and the infusion rates of exogenous AcAc.

Using either stable or radioactive isotopic tracers, one of the problems involved in the determination of ketone body kinetics is the lack of isotopic equilibrium

between acetoacetate and D-β-hydroxybutyrate. This disequilibrium is more evident in situations with low ketone body levels and less apparent when concentrations are raised to high values [3]. A common approach to circumvent this difficulty when using ^{14}C labelled tracers is to calculate the "total ketone body specific activity". We used a similar approach by calculating the "total moles percent enrichment". Such an approach has been criticised on theoretical grounds [20]. It has been suggested that either separate injections of labelled acetoacetate and D-β-hydroxybutyrate at different times or the simultaneous administration of doubly labelled ketone bodies would provide more accurate results. The first possibility has been employed by Cobelli et al. [21], but is limited to steady-state conditions and requires the assumption that the metabolic state of the subject is the same during the two experiments. Preliminary results of studies in dogs using a double label with both stable and radioactive isotopes have been presented recently [22], and suggest that this approach could provide more accurate results. Theoretically, such an approach could also be used by combining the infusions of two differently labelled stable tracers. However, the present experiments, as well as previous studies with ^{14}C labelled tracers, show that reasonable estimates of ketone body kinetics can be performed using a simpler approach. In conclusion, the present method appears useful for studies of ketone body metabolism in humans in both steady- and non steady-state conditions.

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M. Beylot
INSERM U. 197, Faculté de Médecine Alexis Carrel
rue G. Paradin
F-69372 Lyon Cédex 08, France