Determination of ^{14}C radioactivity in ketone bodies: a new, simplified method and its validation

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Summary A rapid and reproducible method for the assay of ^{14}C radioactivity in ketone bodies is described, based on the heat lability of acetoacetate and the ability of β-hydroxybutyrate to quantitatively bind to and elute from an ion exchange resin. Potential interference from ^{14}C activity in the ketone body metabolites (CO_2, bicarbonate and acetone) during infusion of ^{14}C-labeled ketone bodies is eliminated. The method was validated both by in vitro recovery studies and by the determination of rates of appearance of ketone bodies during infusion of exogenous unlabeled β-hydroxybutyrate in dogs. The technique avoids many of the potential sources of imprecision in previous methods and makes in vivo study of ketone body turnover more practical. —**Miles, J. M., M. W. Haymond, R. A. Rizza, and J. E. Gerich.** Determination of ^{14}C radioactivity in ketone bodies: a new, simplified method and its validation. *J. Lipid Res.* 1980. **21:** 646–650.

Supplementary key words β-hydroxybutyrate · acetoacetate · turnover

Ketone bodies (β-hydroxybutyrate and acetoacetate) are important endogenous fuels and may play a role in the regulation of both glucose (1) and protein metabolism (2). At present, it is difficult to study influences on both the production and utilization of ketone bodies in vivo because available methods for the assessment of ^{14}C radioactivity in ketone bodies, which is necessary for isotopic determination of ketone body flux, are tedious and time consuming (3, 4). Herein is described a new method for the assay of ^{14}C radioactivity in ketone bodies, which takes advantage of the heat lability of acetoacetate (AcAc) and the quantitative binding of β-hydroxybutyrate (βOHB) to an ion exchange resin. The method is simple, reliable, and inexpensive.

MATERIALS AND METHODS

Sodium, D,L,β-hydroxybutyrate (analytical grade), and lithium acetoacetate (90–95% purity), as well as all other reagents required for the microfluorometric assays of βOHB and AcAc (5) were obtained from Sigma Chemical Company (St. Louis, MO).

Sodium [1-^{14}C]acetate, sp act 50 mCi/mmol, and sodium L(+)-[U-^{14}C]lactate, sp act 100 mCi/mmol, were obtained from Amersham Corporation. Potassium D(−)-[3-^{14}C]β-hydroxybutyrate, sp act 11 mCi/mmol (^{14}C-βOHB), [2-^{14}C]acetone, sp act 7.5 mCi/mmol and [^{14}C]$NaHCO_3$, sp act 1.2 mCi/mmol, were obtained from New England Nuclear Corporation (Boston, MA). ^{14}C-β-OHB was passed through a Millipore® filter (0.22 μm) into sterile vials, autoclaved, and stored at 4°C. An aliquot of each lot was diluted in normal saline and determined to be pyrogen-free (FDA-approved laboratory) and sterile (bacteria and fungi). When the ^{14}C-β-OHB was analyzed by high pressure liquid chromatography (HPLC), ^{14}C appeared as a single peak that co-migrated with unlabeled sodium D,L-βOHB, indicating greater than 96% purity.

An acetone trap was prepared by saturating a 35-cm filter paper disc with a solution of 2,4-dinitrophenylhydrazine (Eastman Organic Chemicals, Rochester, NY) prepared in boiling ethanol. The filter paper is placed in a 38 × 42 cm aluminum lyophilization chamber capable of accommodating 250 samples. AG1-x8 anion exchange resin and AG50W-x8 cation exchange resin (Bio-Rad Laboratories, Richmond, CA) were each prepared for use by rinsing 10 times with an equal volume of deionized water and were stored at 4°C as a 1:1 (vol/vol) suspension in deionized water. Disposable 1 × 8 cm plastic columns (Quik-Sep columns with plastic filter disk) were obtained from Isolab, Inc. (Akron, OH). Resin suspension (1.5 ml) was added to the columns and rinsed with 2–4 ml of deionized water immediately prior to use.

Scintillation vials (20 ml) were obtained from Curtin Matheson Industries (Houston, TX). Scintillation medium (Aquasol®) was obtained from New England Nuclear Corporation (Boston, MA). Radioactivity was determined in an Isocap/300 liquid scintillation spectrometer (Searle, Des Plaines, IL). Quench correction was performed by external standardization with a ^{133}Ba source.

PROCEDURE

Blood (6–8 ml) is collected in iced heparinized tubes during infusion of [^{14}C]βOHB (or [^{14}C]AcAc). After centrifugation at 4°C for 10 min, 3.5 ml of plasma is deproteinized by addition of an equal volume of 0.5 M perchloric acid. The resultant protein-free filtrate is neutralized to pH 7–8 with 3.7% KOH. One-

Abbreviations: βOHB, β-hydroxybutyrate; AcAc, acetoacetate; HPLC, high pressure liquid chromatography.

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half ml is taken for microfluorometric enzymatic analysis of βOHB and AcAc. The remainder is transferred in duplicate into scintillation vials as follows: Vial A (1.0 ml) and Vial B (2.0 ml). All of the above procedures are carried out on ice to minimize decarboxylation of AcAc. Vial B is placed in a boiling water bath for 20 min; both sets of vials are then frozen at −80°C.

Vial A is placed in a lyophilization chamber containing filter paper impregnated with 2,4dinitrophenylhydrazine, and its contents are taken to dryness at −20°C; the residue is resuspended in 1 ml of deionized water, and 10 ml of liquid scintillation medium is then added. The contents of Vial B are lyophilized as above; the residue is resuspended in 2.0 ml deionized water. One (1.0) ml of this is applied to the ion exchange column. Ten ml of liquid scintillation medium is then added to the remaining solution in Vial B. The column to which 1.0 ml of the contents of Vial B had been applied is rinsed three times with 0.5 ml deionized water, and the rinse is discarded. The column is then eluted with three aliquots (0.6 ml each) of freshly prepared 3 M formic acid into a liquid scintillation vial (Vial C). The contents of this vial are allowed to stand at room temperature for 8 hr, after which 15 ml of liquid scintillation medium is added.

The radioactivity in Vials A, B, and C is determined in a liquid scintillation spectrometer. The difference in radioactivity between Vial A and Vial B is taken as the radioactivity in [^{14}C]AcAc; the radioactivity in Vial C is taken as the radioactivity in [^{14}C]βOHB. Therefore, total ketone body radioactivity ([^{14}C]AcAc + [^{14}C]-βOHB) is the dpm in Vial A minus the dpm in Vial B plus the DPM in Vial C. The dpm are corrected for dilution (dpm/ml plasma) and divided by the concentration of βOHB plus AcAc (μmol/ml plasma) to give total plasma ketone body specific activity (SA).

Recovery of unlabeled AcAc and βOHB and ^{14}C-labeled βOHB

To evaluate potential losses of AcAc and βOHB during the assay procedure, unlabeled AcAc and D,L-βOHB were added to plasma. Samples of plasma (2 ml) were deproteinized, neutralized, and lyophilized to dryness as described above. The residue was resuspended in a volume of water equal to the original filtrate volume and assayed microfluorometrically for βOHB and AcAc. Recoveries of AcAc and βOHB were 100 ± 1% (range, 94–107) and 99 ± 1% (range, 90–103), respectively (**Fig. 1**). These results indicate that both AcAc and βOHB were stable through the lyophilization step of the assay procedure. When samples were heated in boiling water for 20 min prior to lyophilization, recovery of AcAc was

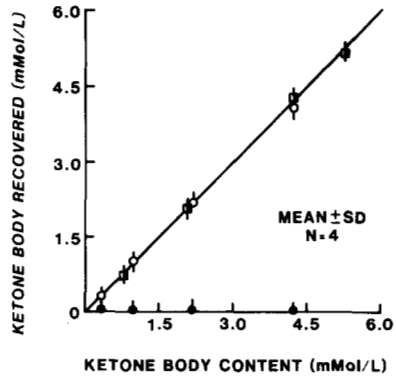

Fig. 1. Recovery of β-hydroxybutyrate and acetoacetate after heating (100°C for 20 min) and lyophilization. ○, AcAc (lyophilized); ●, AcAc (heated and lyophilized); □, βOHB (lyophilized); and ■, βOHB (heated and lyophilized).

1.0 ± 0.3% (range, 0–1.8) and recovery of βOHB was 101 ± 1% (range, 96–110) (Fig. 1). These results indicate essentially complete decarboxylation of AcAc and heat stability of βOHB during the boiling step.

To determine the recovery of βOHB from the AG1-X8 ion exchange resin, [^{14}C]βOHB was added to plasma and the sample was deproteinized, heated, and lyophilized as described above. The residue was suspended in deionized water, and an aliquot (1.0 ml) was applied to the ion exchange column. The column was then rinsed and eluted as outlined above. Recovery of [^{14}C]βOHB was 98.7 ± 0.3% (range, 97.8–100.0). This quantitative binding and subsequent elution of βOHB indicated that ion exchange chromatography was an effective method for isolation of [^{14}C]βOHB.

Assessment of potential interference by ^{14}C-labeled bicarbonate, acetone, lactate, acetate, and amino acids

The major metabolic products of [^{14}C]βOHB which might interfere with the determination of ketone body specific activity by this method are [^{14}C]acetone, [^{14}C]-HCO_3^- and $^{14}CO_2$. [^{14}C]acetone was added to plasma to assess the efficiency of the lyophilization step in removing this metabolic intermediate. Initially considerable amounts (10–20%) of [^{14}C]acetone remained in the samples after lyophilization and contaminated adjacent unlabeled vials. Inclusion of an acetone trap (2,4-dinitrophenylhydrazine-impregnated filter paper) in the lyophilization chamber prevented carryover of radioactivity and resulted in nearly complete removal of [^{14}C]acetone from sample vials (<2% residual counts).

To determine the potential contamination in the procedure due to radioactivity in HCO_3^- and CO_2,

[^{14}C]$NaHCO_3$ was added to plasma. Seventy percent of the HCO_3^- radioactivity was lost during deproteinization. No additional loss was observed with heating and lyophilization of the neutralized samples. After application to the ion exchange column, 14 ± 1% of the original radioactivity was recovered in the formic acid eluate. When the eluate was allowed to stand at room temperature for 8 hr prior to suspension in scintillation medium, 0.1% of activity remained. Thus, complete removal of potential contamination by [^{14}C]-HCO_3-radioactivity was accomplished by the acidification steps (perchloric acid and formic acid) in conjunction with the 8 hr incubation at room temperature. This also indicates that any potential contamination due to $^{14}CO_2$ is eliminated.

To determine the potential contamination by [^{14}C]-lactate and [^{14}C]acetate as a result of these compounds being generated from the infused [^{14}C]-ketone body, [^{14}C]lactate and [^{14}C]acetate were added to plasma which was then processed as above. Both materials were stable to heat and lyophilization at pH 7, and therefore could not interfere with the determination of [^{14}C]-activity in AcAc. Both compounds, however, bound to the anion exchange resin and could be quantitatively eluted with 3 M HCOOH. Subsequent lyophilization of the acid eluate resulted in <1% recovery of acetate and >98% recovery of lactate. Samples from dogs infused for 6–7 hr with [^{14}C]βOHB (see section on in vivo studies) were applied to the anion exchange resin, eluted with HCOOH, lyophilized, and analyzed by high pressure liquid chromatography using a phosphate buffer system (6). Only 5–6% of the radioactivity appeared in the lactate fraction.

Because lactate and acetate are incompletely resolved in this HPLC system, the extent of labeling of the acetate pool was examined indirectly by determining whether additional counts were present in the lactate-acetate fraction when samples were lyophilized at a neutral pH as compared with samples lyophilized at acid pH. An additional dog was infused for 6 hr with [^{14}C]βOHB at 2 μCi/kg-min to permit a more accurate estimation of the radioactivity in lactate and acetate. Samples from the last 120 min of this infusion period were deproteinized, neutralized, and injected directly onto the HPLC system; a second aliquot was heated, lyophilized, passed over an AG-1x8 resin, eluted with 3 M formic acid, lyophilized again, and subjected to HPLC. The ^{14}C in the lactate-acetate peak was, as before, only 5–6% of the β-OHB cpm with both methods of sample preparation.

Samples from dog studies were also passed over an AG50W-x8 cation exchange resin column to assess labeling of amino acids. Eight to nine percent of nonvolatile counts bound to the resin; there was no loss of activity when the eluate was subjected to heating. Since amino acids do not bind to the AG-1x8 resin, no interference from amino acids in the measurement of either [^{14}C]AcAc or [^{14}C]βOHB would be expected. Thus it appears that most of the contamination in this method results from the labeling of the lactate pool; this would introduce, at most, a 3–4% error in the calculation of total ketone body specific activity.

In vivo studies

The ability of the method to accurately estimate ketone body flux in vivo was evaluated by studies in three unanesthetized fasted (6–8 hr) mongrel dogs (10–20 kg). A 16-gauge catheter was placed in the inferior vena cava via a hind limb for blood sampling. A primed (0.3 μCi) continuous (0.01 μCi/kg-min) infusion of [^{14}C]βOHB was begun via an 18-gauge catheter which had been inserted in a forelimb vein. Ninety minutes were allowed to achieve isotopic steady state, and samples were obtained at −30, −20, −10, and 0 min for basal turnover rates. At 0 min, sodium D-β-hydroxybutyrate (as the racemic mixture) was infused at a rate of 30 μmol/kg-min for 180 min; subsequently the rate was reduced to 15 μmol/kg-min for an additional 180 min. The administration rate of the unlabeled β-hydroxybutyrate was confirmed by direct microfluorometric enzymatic analysis of the infusate. The infusion rate of the [^{14}C]βOHB was determined by multiplying the rate of infusion (ml/min) by the ^{14}C radioactivity/ml infusate. Ketone body appearance rates were calculated utilizing the nonsteady-state equations of Wall et al (7) as modified by Cowan and Hetenyi (8) (see Appendix). The acute volume of distribution used for these calculations was 23% of body weight; this had been previously determined by bolus injection of [^{14}C]β-OHB in other dogs and is in close agreement with the findings of other workers (9, 10). A "pool fraction" correction was made by multiplying the volume of distribution by 0.8 as suggested by Sonnenberg and Keller (10).

The plasma ketone body concentration (AcAc + βOHB) was 0.046 ± 0.005 μmol/ml in the basal period; it increased to 1.322 ± 0.23 μmol/ml after 3 hr of infusion of D-β-OHB at a rate of 30 μmol/kg-min and decreased to 0.559 ± 0.07 μmol/ml when the infusion rate was decreased to 15 μmol/kg-min. AcAc specific activity averaged 43 ± 4% of the βOHB specific activity; similar isotopic disequilibrium has been reported by others with the use of both [^{14}C]AcAc and [^{14}C]βOHB (11–13), and provides a rationale for the use of total ketone body (AcAc + βOHB) specific activity in the calculations.

Basal ketone body appearance rates averaged 1.8 ± 0.5 μmol/kg-min (**Fig. 2**). At minute 60 of the 30

μmol/kg-min infusion of unlabeled βOHB, the rate of appearance of total ketone bodies was 28.9 ± 1.5 μmol/kg-min and averaged 29.5 ± 1.8 μmol/kg-min for the remainder of the 180 minute infusion. The infusion rate of the unlabeled βOHB was decreased to 15 μmol/kg-min; the rate of ketone body appearance decreased and averaged 14.3 ± 0.7 μmol/kg-min during this 180 minute period.

Assuming no suppression of basal ketone body production during the infusion of exogenous ketone body, the present method accounted for 93% of ketone body appearance (endogenous + exogenous) at the 30 μmol/kg-min infusion rate and 85% at the 15 μmol/kg-min infusion rate. However, substantial suppression of endogenous ketone body production during infusion of exogenous ketone bodies has been reported (14); thus these calculations probably underestimate the accuracy of the present method.

DISCUSSION

Available methods for the determination of non-steady-state rates of ketone body appearance in vivo include 1) the measurement of arteriovenous differences across the splanchnic bed (15), an approach which can accurately measure net splanchnic production, but is invasive, and cannot quantitate rates of utilization; and 2) the isotope dilution technique utilizing ^{14}C-labeled AcAc or βOHB (11–14, 16, 17), a method which is less invasive, and can quantitate both production and utilization, but which has required difficult procedures for the accurate determination of specific activity.

The established methods for the determination of ^{14}C radioactivity in ketone bodies are based on those of Van Slyke (4) and Mayes and Felts (3). Both methods require the quantitative conversion of ketone bodies to acetone and the subsequent isolation of [^{14}C]-acetone in $HgSO_4$ or in hydrazine lactate, respectively; these procedures are tedious and time-consuming despite various modifications (11, 12, 14, 16, 17). The present method takes advantage of the heat lability of AcAc and the quantitative binding of βOHB to an ion exchange resin to assess ^{14}C radioactivity in AcAc and βOHB from potentially labeled metabolites of the infused ^{14}C tracer, and does not require enzymatic conversion, correction for incomplete recovery, specialized glassware, or multiple transfers of sample and reagent: factors which make previous methods difficult and are potential sources of imprecision.

Rates of ketone body appearance, determined by the isotope dilution technique in humans undergoing prolonged fasts, have varied greatly. Reichard et al

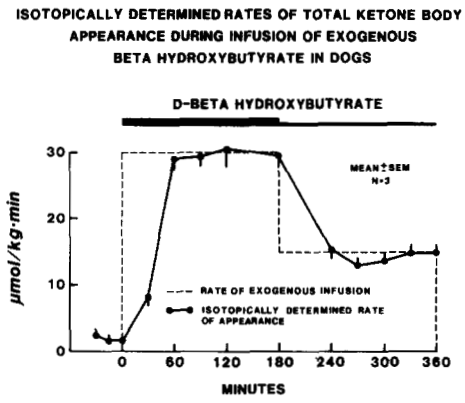

Fig. 2. Rate of appearance of total ketone bodies determined isotopically during infusion of unlabeled D-β-hydroxybutyrate in dogs.

(11) observed ketone body fluxes of 10–11 μmol/kg-min after a 17–24 day fast in obese subjects using the method of Van Slyke (4), whereas Balasse (13) reported rates of 22–23 μmol/kg-min under similar conditions using a modification of the method of Mayes-Felts. To our knowledge the only in vivo validation of either of these methods was performed by Keller, Cherrington, and Liljenquist (12). These investigators demonstrated a close correlation between ketone body production rates in dogs determined by the hepatic vein catheterization technique and those simultaneously determined by an isotopic dilution technique using a modification of the Mayes-Felts method. In the present study, excellent agreement was observed between the isotopically determined rate of ketone body appearance and the rate of exogenous ketone body infusion. Indeed, assuming 33% suppression of endogenous ketone body production during infusion of exogenous ketone body as has been reported by Balasse (14), the present method accounted for in excess of 90% of the rate of appearance of the infused ketone body; approximately half of this underestimate can be explained by a small amount of radioactivity present in lactate.

In conclusion, the method described herein permits a large number of samples to be processed with relative ease, avoids some of the time consuming steps of previous methods which are potential sources for imprecisions and provides a reliable and accurate means for determining ketone body fluxes in vivo.

APPENDIX

Modified Steele's equations:

$$R_a = \frac{F - \frac{(P_{t_1} + P_{t_2})}{(2)} \frac{(SA_{t_2} - SA_{t_1})}{(t_2 - t_1)}}{\frac{(SA_{t_1} + SA_{t_2})}{(2)}} \qquad 1)$$

$$R_d = R_a - \frac{(P_{t_2} - P_{t_1})}{(t_2 - t_1)} \quad 2)$$

Where R_a is rate of appearance (μmol/kg-min); R_d is rate of disappearance; F is tracer infusion rate (DPM/min); P is substrate pool size = body weight (kg) × volume of distribution (ml/kg) × pool fraction × substrate concentration (μmol/ml); SA is specific activity; and t_1 and t_2 represent two different time points.

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