Note

Journal of Labelled Compounds and Radiopharmaceuticals

Received 2 November 2007, Revised 28 January 2008, Accepted 29 January 2008 Published online 26 March 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI: 10.1002/jlcr.1504

Automated synthesis of ^{11}C-β-hydroxybutyrate by enzymatic conversion of ^{11}C-acetoacetate using β-hydroxybutyrate dehydrogenase

Sébastien Tremblay,[a,c*] René Ouellet,[b] François Bénard,[b,c] and Stephen C. Cunnane[a,d,e]

1-[^{11}C]-β-hydroxybutyrate was produced by conversion from 1-[^{11}C]-acetoacetate using (D)-β-hydroxybutyrate dehydrogenase in the presence of nicotinamide adenine dinucleotide with purification by ion exchange column chromatography. Radiochemical yield at the end of the synthesis was 10% for a total synthesis time of 36 min. High-performance liquid chromatography analysis showed $\leqslant$4% impurities, principally unconverted acetoacetate. Residual tetrahydrofuran (34 $\pm$ 11 ppm) and ethanol (77 $\pm$ 30 ppm) were well under the tolerable limits for human studies.

Keywords: ketone bodies; acetoacetate; β-hydroxybutyrate; β-hydroxybutyrate dehydrogenase; PET; [^{11}C]CO_2

Introduction

When glucose is low, ketone bodies (ketones) are the preferred source of energy for extrahepatic cells, particularly for the human brain where they can supply up to 70% of its energy requirements.[1] Ketones are produced in the liver by fatty acid β-oxidation and refer to three compounds: acetone, acetoacetate, and 3-(D)-β-hydroxybutyrate (hereafter β-hydroxybutyrate). In many tissues, acetoacetate and β-hydroxybutyrate are interconvertible by the action of β-hydroxybutyrate dehydrogenase, whereas acetone is formed by the spontaneous or enzymatic decarboxylation of acetoacetate. In addition to being brain fuels, ketones are substrates for amino acid, fatty acid, and cholesterol synthesis, and are implicated in processes such as myelin formation. Furthermore, at least in the suckling rat, ketones are better substrates for brain lipid synthesis than glucose.[2,3]

Ketones have neuroprotective effects in isolated neurons,[4,5] in children with refractory epilepsy,[6] in models of ischemic stroke,[7] and against oxidative stress.[8] Using positron emission tomography (PET) with ^{18}fluorodeoxyglucose, lower brain uptake of glucose has been reported in elderly humans.[9,10] PET studies also show a significant decrease in brain glucose uptake in Alzheimer's disease.[11,12] Short-term improvement in cognitive function in Alzheimer's subjects has been demonstrated using a ketogenic meal;[13] therefore, it is possible that ketones might be useful in overcoming defective brain glucose transport in Alzheimer's disease. Hence, studies supporting the neurotherapeutic potential of ketones are emerging, but further exploration of the human brain's ability to utilize ketones is needed.

An automated synthesis of 1-[^{11}C]-β-hydroxybutyrate[14] and its use for human brain PET imaging have been reported.[15–17] This method requires synthesis conditions that involved conversion of carbon dioxide to ammonium cyanide in the preparation of 1-[^{11}C]-β-hydroxybutyrate. We have previously reported a one pot automated method synthesis of 1-[^{11}C]-acetoacetate.[18] We show here that 1-[^{11}C]-acetoacetate can quickly and easily be reduced to 1-[^{11}C]-β-hydroxybutyrate by D(R)-β-hydroxybutyrate dehydrogenase (Scheme 1), using coenzyme nicotinamide adenine dinucleotide (NADH) as the electron donor. Although normally a reversible reaction, at the appropriate pH, β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) stereospecifically and regioselectively catalyzes the reduction of acetoacetate to the D (R) isomer of β-hydroxybutyrate.[19]

Results and discussion

The synthesis and carboxylation of the isopropenolate anion were achieved under dry and inert atmosphere to avoid reaction with moisture and atmospheric carbon dioxide. Based on the

[a]*Research Center on Aging, Sherbrooke, Que., Canada*

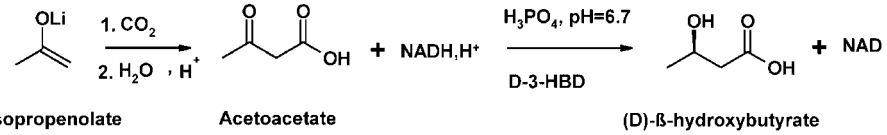
[b]*Sherbrooke Molecular Imaging Centre, Etienne-LeBel Clinical Research Centre, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Que., Canada*

[c]*Department of Nuclear Medicine and Radiobiology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Que., Canada J1H 5N4*

[d]*Department of Medicine, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Que., Canada J1H 5N4*

[e]*Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Que., Canada J1H 5N4*

**Correspondence to: Sébastien Tremblay, Research Center on Aging, Health and Social Services Center, Sherbrooke University Geriatric Institute, 1036 Belvedere Street South, Sherbrooke, Que., Canada J1H 4C4.*
E-mail: Sebastien.Tremblay@USherbrooke.ca



Scheme 1. Radiolabeled synthesis of (D)-β-hydroxybutyrate by stereo-selective reduction of acetoacetate by (D)-β-hydroxybutyrate dehydrogenase (D-3-HBD).

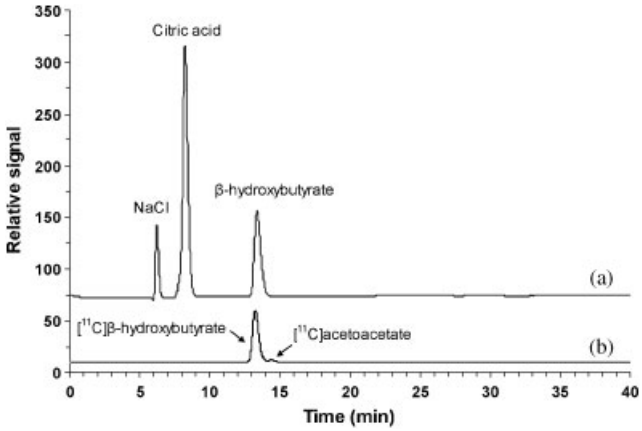


Figure 1. HPLC separation of the product of the radioactive synthesis. Chromatograms from the refractive index detector (a) and the radioactive detector (b) are from a sample spiked with β-hydroxybutyrate cold standard.

[^{11}C]CO_2 delivered to the reaction vessel, the yield of 1-[^{11}C]-β-hydroxybutyrate was 10% (1.9 $\pm$ 0.2 GBq; mean $\pm$ SEM; $n = 10$) at the end of the synthesis and after decay correction was 30%. Synthesis time from the end of bombardment was 36 min. By high-performance liquid chromatography (HPLC), the radiochemical purity of the 1-[^{11}C]-β-hydroxybutyrate was 96.1 $\pm$ 0.5% ($n = 10$) and it co-eluted with the cold β-hydroxybutyrate internal standard with a retention time of 13.2 min. After the synthesis, the main contaminants were identified as unconverted 1-[^{11}C]-acetoacetate (3.6 $\pm$ 0.5%; $n = 10$) at a retention time of 14.5 min, and carbonate ($\geqslant$1%) a retention time of 19.1 min (Figure 1). No other compounds were detected by radioactive or refractive index detector during a 60 min HPLC run. At graded pH from 6.7 to 6.9, no significant differences were observed in the conversion rate of 1-[^{11}C]-acetoacetate to 1-[^{11}C]-β-hydroxybutyrate by enzyme concentrations between 10 and 50 U (Table 1). By HPLC analysis of the final product, the conversion was observed to be mostly affected by pH; if the pH was 7.2–7.6, conversion to β-hydroxybutyrate was reduced to 87.2 $\pm$ 2.5% ($n = 7$) compared with 96.1 $\pm$ 0.5% ($n = 10$) at pHs between 6.7 and 6.9 (Table 1). The stereospecificity of the reduction in acetoacetate to the D (R) isomer of β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase was reported by Passingham and Barton;[19] therefore, we did not reconfirm the enantiomeric purity for this radiosynthesis.

In the final product, some residual ethanol (34 $\pm$ 11 ppm) from the sterilization procedure and some residual tetrahydrofuran (THF) (77 $\pm$ 30 ppm) from the reaction solvent were present, but both were well under the permitted limit in Canada for injection into humans (both 8000 ppm or 50 mg). The final solution ready for injection was at pH 5.5, which is also acceptable for studies *in vivo*. Thioglycollate, Tripticase Soy Broth, and Limulus Amebocyte Lysate pyrogen tests were consistently negative ($n = 3$), demonstrating that the samples were free of bacteria and pyrogens (see Reference[18] for sterilization and bacteriological purity procedures). The Bradford protein test was negative for β-hydroxybutyrate dehydrogenase protein in the product vials (limit of sensitivity of this assay is 1 μg/ml).

Experimental

Chemicals and reagents

Methyllithium (1.6 M in ether), isopropenyl acetate (99%), Dowex 50WX8-100 (cation exchange resin), sodium chloride, β-hydroxybutyrate, and acetoacetate lithium salt were obtained from Aldrich (Oakville, Ont., Canada). Citric acid monohydrate and citric acid trisodium salt were supplied by Fisher Scientific (Whitby, Ont., Canada). Phosphoric acid (85%), anhydrous tetrahydrofuran (THF, H_2O $<$0.005%), sulfuric acid ($\geqslant$95%), and sodium hydroxide pellets were purchased from Fluka (Oakville, Ont., Canada). AG 1-X8 200–400 mesh (anion exchange; 1 KDa size exclusion resin) and Bradford reagent were purchased from Bio-Rad (Mississauga, Ont., Canada). β-hydroxybutyrate dehydrogenase (D-3-HBD) was purchased from Toyobo Ltd. (Tokyo, Japan).

The composition of the sterile isotonic citrate buffer (eluting solution for the radiolabel) was previously described,[18] but the volume was changed to 7 ml (each aliquot of buffer contained 65 mg citric acid, 70 mg citric acid trisodium salt, and 31.5 mg sodium chloride at pH 4).

For the enzyme stock solution, 1000 U (158 U/mg) of β-hydroxybutyrate dehydrogenase was resuspended in 5 ml of 0.2 M K_2HPO_4 at pH 7.5 and kept at 4°C until use. Before each synthesis, a fresh solution consisting of 10–50 U of β-hydroxybutyrate dehydrogenase and 4–6 mM NADH was adjusted to a volume of 2.95 ml with water and kept at 4°C until use.

The anion exchange and exclusion weight column was loaded as previously described,[18] except that 250 mg of AG 1-X8 resin was used and the column was conditioned with 10 ml 1 M NaOH followed by rinsing with sterile water until a neutral pH was reached. To prepare the cation exchange column, 500 mg of Dowex 50WX8-100 was utilized and washed with 10 ml of sterile water.

Synthesis of the lithium isopropenolate precursor

Synthesis of the isopropenolate precursor was as described previously.[18] In brief, the isopropenolate anion precursor was produced at -80°C, under anhydrous conditions, by the addition of 6.25 mmol isopropenyl acetate to 12.5 mmol methyllithium with stirring for 1 h and dilution in THF to 0.37 mmol/ml. The isopropenolate anion solution was replaced by a fresh one after 1 month at -80°C.

Synthesis module

The synthesis module was as previously described[18] with slight modification. In brief, compared with the acetoacetate synthesis

Table 1. Conversion yield of 1-[^{11}C]-β-hydroxybutyrate from 1-[^{11}C]-acetoacetate by β-hydroxybutyrate dehydrogenase as estimated by HPLC analysis on radio-labeled compounds, during which the unconverted substrate was identified as 1-[^{11}C]-acetoacetate

pH Value	Enzyme (Unit)	Yield (%)	Unconverted substrate (%)	*n*
7.6	50	85.4 ± 0.4	14.6 ± 0.4	2
7.2	50	88.5 ± 4.3	9.4 ± 4.3	5
6.9	50	95.3	2.0	1
6.9	20	96.4 ± 0.4	3.6 ± 0.4	2
6.9	10	96.1 ± 0.5	3.7 ± 0.4	4
6.7	10	96.1 ± 0.6	3.9 ± 0.6	3

Percentage values for conversion and unconverted substrate were those actually measured. The *n* represents the number of experiments with errors expressed as mean ± SEM.

unit,[18] vial 1 was replaced with a phosphoric acid solution (125 mM) to hydrolyze unreacted isopropenolate solution and achieve a neutral pH. Vial 2 was filled with the enzyme solution and a heater for the reaction vessel was added.

Description of the labeling procedures

[^{11}C]CO_2 was produced without a carrier as previously described[18] from the nuclear reaction—^{14}N(p,α)^{11}C—by proton bombardment of a 56.6 ml volume aluminium target filled with 99.5% nitrogen/oxygen gas to a pressure of 17.2 bar (250 psi).[18] The irradiation beam was set at 17.6 MeV by a cyclotron (model TR19, Advanced Cyclotron Systems, Richmond, BC, Canada) with a current beam of 25 μA for 7 min (yield 19.5 GBq of [^{11}C]CO_2 at end of bombardment).

After irradiating the target and capture of [^{11}C]CO_2 by the molecular sieve column, the radioactive gas was released by heating the column to 225°C and was transported to the reaction vial with a helium flow of 25 ml/min.[18] The [^{11}C]CO_2 was reacted with 111 μmol isopropenolate anion in 300 μl THF at 4°C. A 5 min reaction was allowed with a slow increase in room temperature to produce the 1-[^{11}C]-acetoacetate (10 min in total for the release and reaction process).[18]

To adjust pH for the enzymatic reaction with the β-hydroxybutyrate dehydrogenase, 1.15–1.75 ml of 125 mM H_3PO_4 was added from vial 1. The enzyme mixture in vial 2 was then added to the reactor and the temperature increased to 40°C for 14 min.

The reaction mixture was then pushed through the DOWEX and AG 1-X8 columns into the waste vial. The newly formed 1-[^{11}C]-β-hydroxybutyrate was washed off the AG 1-X8 column with 10 ml of sterile water from vial 3. The 1-[^{11}C]-β-hydroxybutyrate was then eluted with 7 ml citrate buffer, from vial 4, to the degassing chamber. Two minutes of helium bubbling exhausted any unreacted [^{11}C]CO_2 from the final solution. The 1-[^{11}C]-β-hydroxybutyrate solution was then dispensed out of the synthesis module under helium pressure and sterilized by filtration through a Millex-GS filter (Millipore) into a titanium-shielded vial.

Residual solvents

The presence of unwanted residual solvents in the 1-[^{11}C]-β-hydroxybutyrate preparation was assessed by gas chromatography (GC) as previously described.[18] The GC retention times for THF and ethanol were 1.5 and 2.2 min, respectively.

HPLC separation

The identity of the 1-[^{11}C]-β-hydroxybutyrate produced was confirmed by comparison of its HPLC retention time with that of the commercially available cold analog. As previously described,[18] the HPLC system had the following retention times: NaCl—6.3 min, citrate—8.3 min, β-hydroxybutyrate—13.2 min, acetoacetate—14.5 min, carbonate—19.1 min, ethanol—21.0 min, and THF—31.9 min.

Residual enzyme

The final product was concentrated to 0.8 ml with a centrifugal micro-separation device with a molecular weight cut-off of 10 kDa (Pall Life Sciences, East Hills, NY, USA). A test for protein content was performed by adding 200 μl of Bradford reagent for 5 min, followed by UV absorbance measurement at 595 nm (Beckman, DU530, Fullerton, CA, USA), with comparison to a standard curve made from bovine serum albumin (Bio-Rad) for quantification.

Conclusion

This procedure permits the synthesis of 1-[^{11}C]-β-hydroxybutyrate in sufficient purity and yield for human PET studies. It uses gentler synthesis conditions compared with the previously described ammonia cyanide procedure,[14] and does not require time-consuming HPLC purification. Furthermore, with slight modifications, it could be adapted to the commercially available 1-[^{11}C]-acetate synthesis module. Analogous to our ongoing work with 1-[^{11}C]-acetoacetate,[20,21] 1-[^{11}C]-β-hydroxybutyrate can be used to monitor ketone uptake by the human or rodent brain under conditions that may affect overall or regional brain ketone metabolism, including controlled mild–moderate ketosis, healthy aging, and neurodegenerative diseases.

Acknowledgement

The authors thank Marcel Turcotte for sterility testing and citrate solution preparation, the cyclotron operator, Serge Rodrigue, Advanced Cyclotron Systems for the new [^{11}C]CO_2 target, and the Sherbrooke Molecular Imaging Centre for technical services. The Canadian Foundation for Innovation and the Canada Research Chairs (SCC) program provided financial support.

References

[1] O. E. Owen, A. P. Morgan, H. G. Kemp, J. M. Sullivan, M. G. Herrera, G. F. Cahill Jr., *J. Clin. Invest.* **1967**, *46*, 1589–1595.
[2] R. J. Webber, J. Edmond, *J. Biol. Chem.* **1977**, *252*, 5222–5226.
[3] D. C. DeVivo, M. P. Leckie, H. C. Agrawal, *J. Neurochem.* **1975**, *25*, 161–170.
[4] Y. Kashiwaya, T. Takeshima, N. Mori, K. Nakashima, K. Clarke, R. L. Veech, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 5440–5444.
[5] H. S. Noh, Y. S. Hah, R. Nilufar, J. Han, J. H. Bong, S. S. Kang, G. J. Cho, W. S. Choi, *J. Neurosci. Res.* **2006**, *83*, 702–709.
[6] J. Freeman, P. Veggiotti, G. Lanzi, A. Tagliabue, E. Perucca, *Epilepsy Res.* **2006**, *68*, 145–180.
[7] R. Masuda, J. W. Monahan, Y. Kashiwaya, *J. Neurosci. Res.* **2005**, *80*, 501–509.
[8] D. Y. Kim, L. M. Davis, P. G. Sullivan, M. Maalouf, T. A. Simeone, J. V. Brederode, J. M. Rho, *J. Neurochem.* **2007**, *101*, 1316–1326.
[9] A. Loessner, A. Alavi, K. U. Lewandrowski, D. Mozley, E. Souder, R. E. Gur, *J. Nucl. Med.* **1995**, *36*, 1141–1149.
[10] M. W. Willis, T. A. Ketter, T. A. Kimbrell, M. S. George, P. Herscovitch, A. L. Danielson, B. E. Benson, R. M. Post, *Psychiatry Res.* **2002**, *114*, 23–37.
[11] G. Kalpouzos, F. Eustache, V. de la Sayette, F. Viader, G. Chetelat, B. Desgranges, *J. Neurol.* **2005**, *252*, 548–558.
[12] E. M. Reiman , K. Chen, G. E. Alexander, R. J. Caselli, D. Bandy, D. Osborne, A. M. Saunders, J. Hardy, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 284–289.
[13] M. A. Reger , S. T. Henderson, C. Hale, B. Cholerton, L. D. Baker, G. S. Watson, K. Hyde, D. Chapman, S. Craft, *Neurobiol. Aging* **2004**, *25*, 311–314.
[14] J. O. Thorell, S. Stone-Elander, C. Halldin, L. Widen, *Acta Radiol. Suppl.* **1991**, *376*, 94.
[15] V. Grill, M. Gutniak, O. Bjorkman, M. Lindqvist, S. Stone-Elander, R. J. Seitz, G. Blomqvist, P. Reichard, L. Widen, *Am. J. Physiol.* **1990**, *258*, E813–E820.
[16] G. Blomqvist, J. O. Thorell, M. Ingvar, V. Grill, L. Widen, S. Stone-Elander, *Am. J. Physiol.* **1995**, *269*, E948–E959.
[17] G. Blomqvist, M. Alvarsson, V. Grill, G. Von Heijne, M. Ingvar, J. O. Thorell, S. Stone-Elander, L. Widen, K. Ekberg, *Am. J. Physiol. Endocrinol. Metab.* **2002**, *283*, E20–E28.
[18] S. Tremblay, R. Ouellet, S. Rodrigue, R. Langlois, F. Benard, S. C. Cunnane, *Appl. Radiat. Isot.* **2007**, *65*, 934–940.
[19] B. J. Passingham, R. N. Barton, *Anal. Biochem.* **1975**, *65*, 418–421.
[20] S. Authier, S. Tremblay, C. Dubuc, V. Dumoulon, R. Ouellet, R. Lecomte, S. C. Cunnane, F. Bénard, *Mol. Imag. & Biol.* **2008**, accepted.
[21] M. Bentourkia, S. Tremblay, J. Rousseau, R. Lecomte, S. C. Cunnane, *J. Nucl. Med.* **2008**, resubmitted.

