Enzymatic Determination of Carbon-14-Labeled D-β-Hydroxybutyrate in Biological Samples

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A method for determination of D-β-hydroxybutyrate-specific radioactivity in biological samples is presented. It is based on the following steps: (a) enzymatic conversion of D-β-hydroxybutyrate to acetoacetate and its conversion to phenylhydrazone, (b) quantitative trapping of the phenylhydrazone in SPE C18 columns, (c) elution with ethanol of the phenylhydrazone of acetoacetate followed by radioactivity counting, and (d) estimation of the radioactivity thus trapped compared with that of enzymatically untreated aliquots of the same samples. No interferences from other ^{14}C-labeled materials such as D-glucose, L-alanine, L-glutamine, and L-valine were observed. This inexpensive and high-speed method can be applied in routine multiple estimations of D-β-hydroxybutyrate-specific radioactivity in biological samples in tracer metabolic studies.

INTRODUCTION

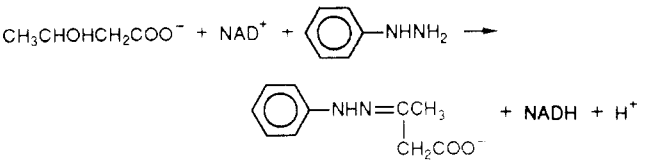
Determination of [^{14}C]-β-hydroxybutyrate radioactivity is of interest in metabolic studies on fatty acid metabolism or ketone body utilization as a metabolic fuel. The study of both the production and the utilization of ketone bodies in vivo is tedious, because the available methods for ^{14}C-labeled ketone bodies radioactivity quantification are considerably time consuming.[1-6]

These methods are based on ketone body decarboxylation in the presence of acids, followed by acetone collection and counting,[1-5] or on the ability of β-hydroxybutyrate to quantitively bind to and elute from an ion-exchange resin.[6]

The use of polymeric porous hydrophobic adsorbing beads to recover hydrophobic compounds from aqueous solutions is a well-documented procedure.[7-13]

Also we had previously applied this property to the estimation of individual amino acid radioactivity in plasma samples,[9] ammonia and urea determination in water samples,[12] enzymatic L-lactate radioactivity,[11] pyruvic-specific radioactivity,[10] and ^{14}C-labeled L-alanine[13] in biological samples.

In this paper we present a method for D-β-hydroxybutyrate-specific radioactivity determination based on the highly specific enzymatic transformation of D-β-hydroxybutyrate to acetoacetate catalyzed by the enzyme D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), coupled to the simultaneous formation of the respective phenylhydrazone derivate as follows:



The procedure is completed by the quantitative trapping of the phenylhydrazone in a simple purification step in SPE C18 columns followed by radioactivity counting of ethanol eluate.

EXPERIMENTAL SECTION

Analytical-grade reagents were used. Labeled material was obtained from Radiochemical Center (Amersham, U.K.) and Dupont Nen (Germany). Biochemical standards and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). The SPE C18 columns were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Samples of rat plasma or standard solutions containing known amounts of potassium, D-(−)-β-[3-^{14}C]-hydroxybutyric acid (from 0.25 to 1.5 mM; final specific radioactivity of 5.4 MBq/mmol) were used. The solutions of U-^{14}C-labeled material used to validate the method contained 0.60 mM L-alanine (16.1 MBq/mmol), 0.50 mM L-glutamine (20.5 MBq/mmol), 6 mM D-glucose (0.9 MBq/mmol), and 0.20 mM L-valine (42.2 MBq/mmol). These solutions were handled under standard safety regulations for the use of radiochemical materials in laboratories.

One milliliter of sample, previously diluted 1:1 with distilled water, was deproteinized with 1 mL of 12% (w/v) perchloric acid. Supernatants were then neutralized with 0.35 mL of 2 N KOH containing 2 N $KHCO_3$ and centrifuged again. Two aliquots of 0.25 mL from each final supernatant were used. The first, A, was incubated with 1 mL of buffer phenylhydrazine-Tris (0.10 M Tris pH = 8.5 with 2.7 mM $EDTANa_2$ and ClH 0.25 N and 1% phenylhydrazine) and 0.10 mL of 14 mM NAD^+, and excess of β-hydroxybutyrate dehydrogenase (10 μL of 5 units/mL). The NADH 334 nm extinction increase was measured and used to calculate the D-β-hydroxybutyrate in samples. The second aliquot, B, was incubated under the same conditions but with no enzyme. Then both aliquots were processed in parallel.

One milliliter of each of the samples was then passed through small 3-mL extraction columns SPE C18 containing 500 mg of sorbent (Phillipsburg, NJ), previously washed with 6 mL ethanol 98% and equilibrated with 6 mL of water; the columns were recycled several times after 98% ethanol washing.

The samples passed through columns with help of the Baker-21 SPE extraction system with vacuum bomb; 1-mL samples were eluted and the column washed with 0.5 mL water. Finally the phenylhydrazone of acetoacetate was eluted with 2 mL of 98% ethanol. The 2 mL of eluated ethanol was mixed with 10 mL of liquid scintillation of OptiPhase 'HiSafe' II (LKB, UK).

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Table I. Distribution and Recoveries of [^{14}C]-β-Hydroxybutyrate Radioactivity and Other ^{14}C-Labeled Materials among Different Fractions Obtained with the Method Described[a]

^{14}C samples (put in column, nmol)	radioactivity (Bq) added a	recovered in water fraction: enzyme treated	recovered in water fraction: enzyme untreated	recovered in ethanol fraction: enzyme treated, A	recovered in ethanol fraction: enzyme untreated, B	difference A − B	Final recovery (A − B) × 100/a
standard solution							
β-hydroxybutyrate (61.5)	332	40 ± 12	323 ± 2	298 ± 2	9 ± 1	280 ± 3	84.3 ± 0.9
β-hydroxybutyrate (123)	663	63 ● 11	650 ± 5	579 ● 11	13 ± 1	566 ± 11	85.4 ± 1.7
β-hydroxybutyrate (246)	1326	144 ± 29	1340 ± 13	1148 ± 22	30 ± 2	1124 ± 22	84.8 ± 1.7
β-hydroxybutyrate (369)	1989	375 ± 5	1940 ● 20	1642 ± 20	30 ± 2	1613 ● 22	81.1 ± 1.5
plasma							
β-hydroxybutyrate (80)	1815	298 ± 40	1790 ± 30	1516 ± 31	24 ± 2	1492 ± 30	82.2 ± 1.7
standard solutions							
D-glucose (640)	590	529 ± 13	536 ± 10	50 ± 1	48 ± 1	2 ± 1	0.37 ± 0.22
L-alanine (64)	1029	999 ± 11	997 ± 16	34 ± 2	31 ± 1	3 ± 2	0.33 ± 0.22
L-glutamine (53)	1089	1038 ± 8	1048 ± 19	45 ± 2	40 ± 1	6 ± 1	0.53 ± 0.13
L-valine (21)	886	705 ± 2	707 ± 4	123 ± 3	127 ± 1	4 ± 3	−0.43 ± 0.28

[a] The values are mean ± sem of four determinations.

The differences between both parallel sample aliquots (A and B) gave the D-β-hydroxybutyrate radioactivity. The D-β-hydroxybutyrate-specific radioactivity could then be calculated.

RESULTS AND DISCUSSION

Table I shows the relation between D-β-hydroxybutyrate radioactivity present in the samples (standard and plasma) in front of radioactivity found in the ethanol eluate with the method described. The steps used in the method can be explained as follows: Firstly the enzymatic conversion of D-β-hydroxybutyrate to acetoacetate and its transformation to phenylhydrazone implies the conversion of a hydrophylic to hydrophobic compound. This change in physical properties is highly specific because of the enzymatic characteristics of the transformation.[14] The latter steps allow the separation of D-β-hydroxybutyrate carbons from other hydrophylic compounds.

The lineal correlation coefficient of added in front of found radioactivity was $r = 0.997$, $n = 16$. The mean percentage of recovery of D-β-hydroxybutyrate was 84% ($n = 16$). No differences were observed between the recoveries from the standard solutions and those from rat plasma (82.2 ± 1.7%) samples. There was a mean loss of 16% of radioactivity in whole derivatization/purification that must be taken into account in the calculation of the original D-β-hydroxybutyrate radioactivity. This figure is very close to that described in the methods based on the formation of phenylhydrazones.[10,11,13]

The specificity of this method is the same as the spectrophotometric enzymatic measurement of D-β-hydroxybutyrate.[14] With respect to possible interference in the D-β-hydroxybutyrate studies from other *p*-hydroxy acids or α-keto acids, it is interesting to note that only the higher homologs of D-β-hydroxybutyrate, 3-hydroxypentanoic and 3-hydroxyhexanoic acids, could also react but at much slower rates,[15] and these compounds are not present in biological samples. Other possible interferences from non-*p*-hydroxy acids have been ruled out in the present study. The recovery of the other labeled materials is also shown in Table I. No interferences from D-glucose, L-glutamine, L-valine, and L-alanine were observed using our conditions in concordance with the specificity of β-hydroxybutyrate dehydrogenase for this substrate and its reactivity with phenylhydrazine.

In addition this method is as simple as to be able to be applied to large numbers of samples for the determination of D-β-hydroxybutyrate radioactivity in tracer metabolic studies.

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