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L-3-Hydroxybutyrate in Rat Tissues

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Abstract

L-3-Hydroxybutyrate (L-3HB), the enantiomer of D-3-hydroxybutyrate (D-3HB), has traditionally been regarded as the “unnatural” ketone body in mammals, although there is suspicion that it is a more-favorable energy fuel for mammalian tissues compared to D-3HB. In the present study, we prove that L-3HB is an original substance in rat serum by applying fluorescence derivatization and a column-switching high-performance liquid chromatography (HPLC) system as the analysis technique. Racemic 3HB in rat serum derivatized using 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) was first separated by an ODS column, and was further confirmed by verifying the disappearance of the racemic 3HB peak after pretreating rat serum with D-3-hydroxybutyryl dehydrogenase (D-3HB dehydrogenase). A switching valve was used to simultaneously introduce isolated racemic 3HB to the enantiomeric separation by two CHIRALCEL OD-RH columns connected in tandem. An L-isomer was found to accompany the D-isomer, which were quantified to be 3.98 (3.61%) and 106.20 µM (96.39%), respectively. Using the present analytical method, the dubious interpretation of the existence of L-3HB was clarified.

Keywords: *L-3-Hydroxybutyrate, Derivatization, NBD-PZ, Enantiomeric separation, Cellulose-based chiral column, Column-switching HPLC.*

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Traditionally, ketone bodies in mammals, which are formed via β-oxidation of fatty acids in the liver, have been thought to consist of acetoacetate, D-3HB, and acetone [1]. The enantiomer of D-3HB, L-3HB, was considered to be an “unnatural” ketone body and to be absent under physiological conditions. However, L-3HB as well as D-3HB can be incorporated into hepatic lipids, brain proteins, and amino acids during the developmental period of neonatal rats [2, 3]. In

addition, L-3HB was shown to be a more-favorable substrate than other ketone bodies for sterol and fatty acid synthesis in the brain, spinal cord, and kidney [4].

This superior utilization of L-3HB was noted in several investigations which attempted to identify related enzymes responsible for L-3HB metabolism, or its original source. Three enzymes, including L-3-hydroxybutyryl CoA dehydrogenase, L-3-hydroxybutyryl CoA deacylase, and L-3-hydroxybutyryl CoA ligase, were proposed by Reed and Ozand [5] to be required for the production and utilization of L-3HB. The liver, heart, and skeletal muscle were shown to contain substantial activities of L-3-hydroxybutyryl CoA dehydrogenase, and this enzyme in the liver was suggested to function in the production of L-3HB. On the contrary, Lincoln *et al.* [6] showed that L-3HB was not metabolized by a dehydrogenase in the rat liver, but mostly via mitochondrial activation. The L-3HB formation pathway, which is palmitic acid incorporated with L-3-hydroxybutyryl CoA deacylase, was also discounted by Scofield *et al*. [7] who failed to detect labeled L-3HB after the liver was perfused with ^{14}C-labeled palmitic acid.

Yet a series of conflicting results exists; the sensitivity of the radiotracer instruments used in the above studies may be the key factor leading to such controversy. Swiatek *et al.* [2] demonstrated that if L-3HB existed in a low steady-state concentration of 5% less than D-3HB, it would be beyond the limit of detection. Indeed, in patients with medium-chain acyl-CoA dehydrogenase deficiency or β-ketothiolase deficiency, L-3HB was found to comprise a minor amount of about 3%-5% of the total racemic 3HB by gas chromatography-mass spectrometry [8], and the proportion of the D- and L-isomers might vary with different diseases. Although this D:L ratio variation seemed to imply altered selectivity and activity of enzymes leading to 3HB, D- and L-3HB being undetectable in the control samples, quantitation of D- and L-3HB was not performed. Therefore, the present study aimed to develop a practical method for identification and quantitation of L-3HB to clarify the dubious interpretation of the existence of L-3HB in mammals.

In addition to the radiotracer detection method, one of the widely used measurements for 3HB is the utilization of D-3HB dehydrogenase, which transforms D-3HB to acetoacetate in the presence of NAD, and then the resultant NADH can be detected by absorption [9-11]. In contrast to D-3HB dehydrogenase, it is controversial to proceed with an enzymatic assay of L-3HB due to the absence of a well-known enzyme specifically responsible for L-3HB catabolism. Additionally, it is difficult to sensitively determine D- and L-3HB with UV detection because of their low absorptivities in the effective wavelength region. To overcome these shortcomings, we employed a fluorimetric method for L-3HB using HPLC following precolumn fluorescence derivatization. Two fluorogenic reagents, 4-(*N*,*N*-dimethyl-aminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole (DBD-PZ) and 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), were chosen for derivatization, for they readily react with the carboxylic group of D- and L-3HB [12, 13]. Another advantage is their long emission wavelengths, so that a great proportion of the substances will be sheltered from overlapping objective peaks. Enantiomeric separation of D- and L-3HB was carried out by HPLC using polysaccharide-based (cellulose or amylose) or phenylcarbamoylated β-CD chiral columns. As a result, the achieved enantiomeric separation was applied to analyze D- and L-3HB in rat serum, both of which were previously separated by an ODS column in the reversed-phase mode. Simultaneous determination of D- and L-3HB in rat serum using column-switching HPLC employing ODS and a chiral column after pre-column derivatization is described in this paper.

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[1] A.L. Lehninger, D.L. Nelson, M.M. Cox, Principles of Biochemistry. *Second Edition*, Worth Publishers, New York, 1993.

[2] K.R. Swiatek, G.J. Dombrowski, K.L. Chao, Biochem. Med. 31 (1984), 332-346.

[3] K.R. Swiatek, G.J. Dombrowski, K.L. Chao, H.L. Chao, Biochem. Med. 25 (1981), 160-167.

[4] R.J. Webber, J. Edmond, J. Biol. Chem. 252 (1977), 5222-5226.

[5] W.D. Reed, P.T. Ozand, Arch. Biochem. Biophys. 205 (1980), 94-103.

[6] B.C. Lincoln, C. Des Rosiers, H. Brunengraber, Arch. Biochem. Biophys. 259 (1987), 149-156.

[7] R.F. Scofield, P.S. Brady, W.C. Schumann, K. Kumaran, S. Ohgaku, J.M. Margolis, B.R. Landau, Arch. Biochem. Biophys. 214 (1982), 268-272.

[8] M. Heil, F. Podebrad, E. Prado, T. Beck, A. Mosandl, A.C. Sewell, H. Böhles, W. Lehnert, J. Chromatogr. B Biomed. Sci. Appl. 739 (2000), 313-324.

[9] D.H. Williamson, J. Mellanby, H.A. Krebs, Enzymic determination of D(-)-b-hydroxybutyric acid and acetoacetic acid in blood, Biochem. J. 82 (1962), 90-96.

[10] J.M. Stephens, M.J. Sulway, P.J. Watkins, Diabetes 20 (1971), 485-489.

[11] M. Fulop, V. Murthy, A. Michilli, J. Nalamati, Q. Qian, Arch. Intern. Med. 159 (1999), 381-384.

[12] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu, K. Imai, J. Chromatogr. A 588 (1991), 61-71.

[13] K. Imai, S. Uzu, S. Kanda, W.R.G. Baeyens, Anal. Chim. Acta. 290 (1994), 3-20.

[14] H. Ichihara, T. Fukushima, K. Imai, Anal. Biochem. 269 (1999), 379-385.

[15] X. Guo, T. Fukushima, F. Li, K. Imai, Analyst

127 (2002), 480-484.
[16] P.A. Ruell, G.C. Gass, Ann. Clin. Biochem. 28 (1991), 183-184.
[17] T. Fukushima, T. Santa, H. Homma, S.M. Al-Kindy, K. Imai, Anal. Chem. 69 (1997), 1793-1799.
[18] E. Yashima, Y. Okamoto, Bull. Chem. Soc. Jpn. 68 (1995), 3289-3307.
[19] H.Y. Aboul-Enein, I. Ali, G. Gübitz, C. Simons, P.J. Nicholls, Chirality 12 (2000), 727-733.
[20] X. Yang, T. Fukushima, T. Santa, H. Homma, K. Imai, Analyst 122 (1997), 1365-1369.
[21] H.Y. Aboul-Enein, I. Ali, J. Biochem. Biophys. Methods 48 (2001), 175-188.
[22] S. Al-Kindy, T. Santa, T. Fukushima, H. Homma, K. Imai, Biomed. Chromatogr. 12 (1998), 276-280.

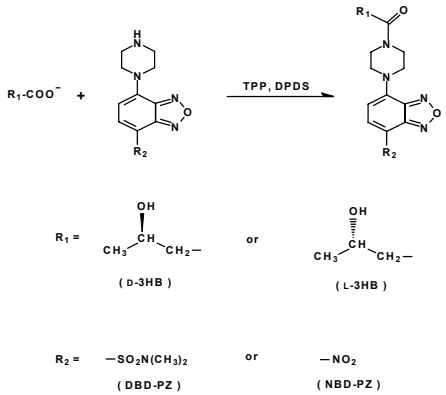

Fig 1. Derivatization scheme of D- and L-3HB with DBD-PZ or NBD-PZ. TPP and DPDS were used as the condensing agents.

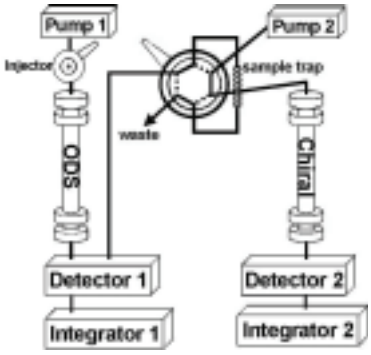

Fig 2. HPLC system with a column-switching valve used in this study. The six-port valve positions A and B are represented by the solid and dotted lines, respectively. A TSKgel ODS-80Ts column was used as the ODS column, and AD-RH, OD-RH, phCD, and tandem OD-RHs were used for the chiral columns. The mobile phase for pump 1 was MeOH/H_2O (33/67) at a flow rate of 0.7 ml/min, and MeOH/H_2O (80/20) or MeCN/H_2O (40/60) for pump 2 at a flow rate of 0.3 ml/min. Detectors 1 and 2 were both set at 547 nm for fluorometric detection and 491 nm for the excitation wavelength.

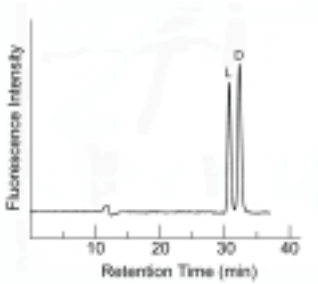

Fig 3. Enantiomeric separation of standard D- and L-3HB derivatized with NBD-PZ by two OD-RHs connected in tandem. As indicated, the L-3HB derivative was eluted before D-3HB, and the Rs value was 1.60.

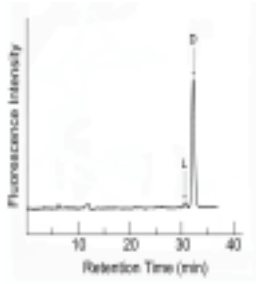

Fig 4. Simultaneous analysis of L-3HB in rat serum. (A) Rat serum derivatized with NBD-PZ was eluted by the TSKgel ODS-80Ts column and the chromatogram was obtained from Integrator 1. The retention times of the total racemic 3HB derivative and the I.S. were about 26 min and 43 min, respectively. (B) Enantiomeric separation of D- and L-3HB derivatives isolated from rat serum. The tandem OD-RHs was the selected chiral column to perform an efficient separation, and the chromatogram was from Integrator 2. L-3HB is shown on the chromatogram and can be identified to be the part of the 3HB composition in the rat.

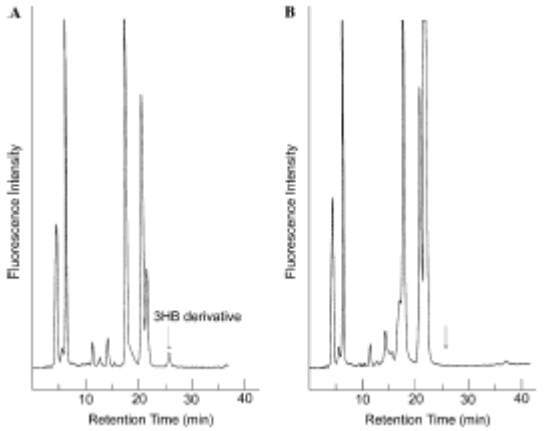

Figure 5. Confirmation of D-3HB by verifying the disappearing peak on chromatograms from Integrator 1 with D-3HB dehydrogenase pretreatment. The peak representing the racemic 3HB derivative on chromatograms could be found without pretreatment with D-3HB dehydrogenase (A), but it disappeared in the presence of the enzyme since its major constituent, D-3HB, was converted to acetoacetate (B). The arrow in (B) indicates the retention time of the racemic 3HB derivative.

Table 1. Capacity factor (k'), separation factor (α), and resolution factor (Rs) of enantiomeric separation of D- and L-3HB derivatized using NBD-PZ or DBD-PZ by chiral columns including AD-RH, phCD, OD-RH, and tandem OD-RHs with mobile phase compositions of MeOH/H_2O (80/20) and MeCN/H_2O (40/60).

	NBD-PZ				DBD-PZ			
	$k'_{L\text{-}3HB}$	$k'_{D\text{-}3HB}$	α	Rs	$k'_{L\text{-}3HB}$	$k'_{D\text{-}3HB}$	α	Rs
MeOH/H_2O (80/20)								
AD-RH	9.91	14.25	1.44	1.99	5.28	5.25	1	—
phCD	4.26	4.65	1.09	1.00	1.58	1.72	1.11	1.13
OD-RH	5.40	7.36	1.31	3.46	2.68	2.63	1	—
Tandem OD-RHs	5.76	7.99	1.31	3.50	3.13	3.13	1	—
MeCN/H_2O (60/40)								
AD-RH	2.65	2.98	1.11	0.77	1.32	1.32	1	—
PhCD	2.97	2.29	1.04	0.73	1.68	1.75	1.07	0.98
OD-RH	1.35	1.47	1.09	1.12	1.28	1.23	1	—
Tandem OD-RHs	1.38	1.65	1.09	1.60	1.31	1.31	1	—

Table 2. Concentrations and percentages of total racemic, D-, and L-3HB in rat serum (n = 5).

	Concentration (μM)	Percentage (%)
Total racemic 3HB	110.17 ± 22.75	100
D-3HB	106.20 ± 22.65	96.39
L-3HB	3.98 ± 0.78	3.61

Concentrations are expressed as the mean ± SD.

Table 3. Precision and accuracy of L-3HB measurements (n = 5).

	L-3HB added (μM)			
	0	2.00	4.00	6.00
Intraday				
Measured (μM)	3.40	5.43	7.71	9.85
RSD (%)	3.24	6.87	4.10	4.16
Recovery (%)	—	101.60	107.90	107.54
Interday				
Measured (μM)	4.75	6.85	8.65	11.02
RSD (%)	10.52	5.31	10.86	13.10
Recovery (%)	—	104.88	97.30	104.38

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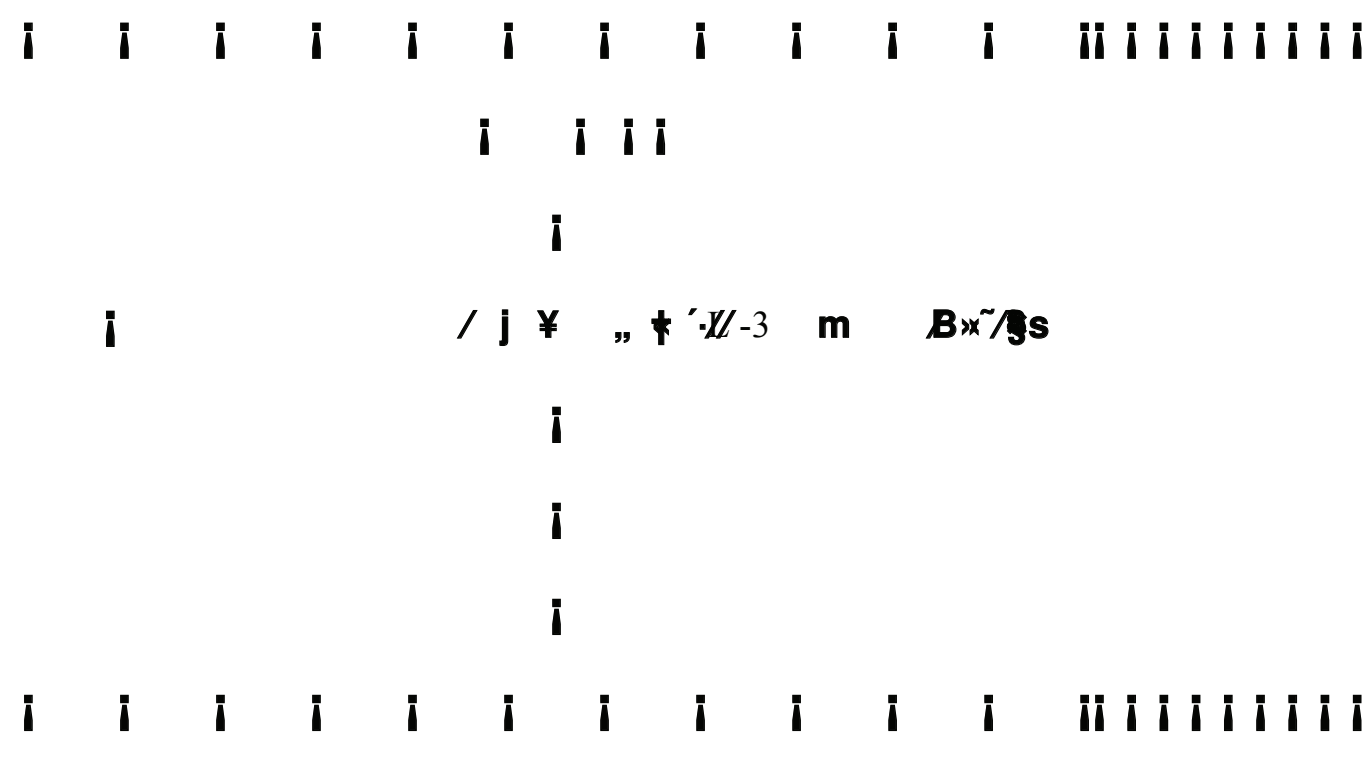

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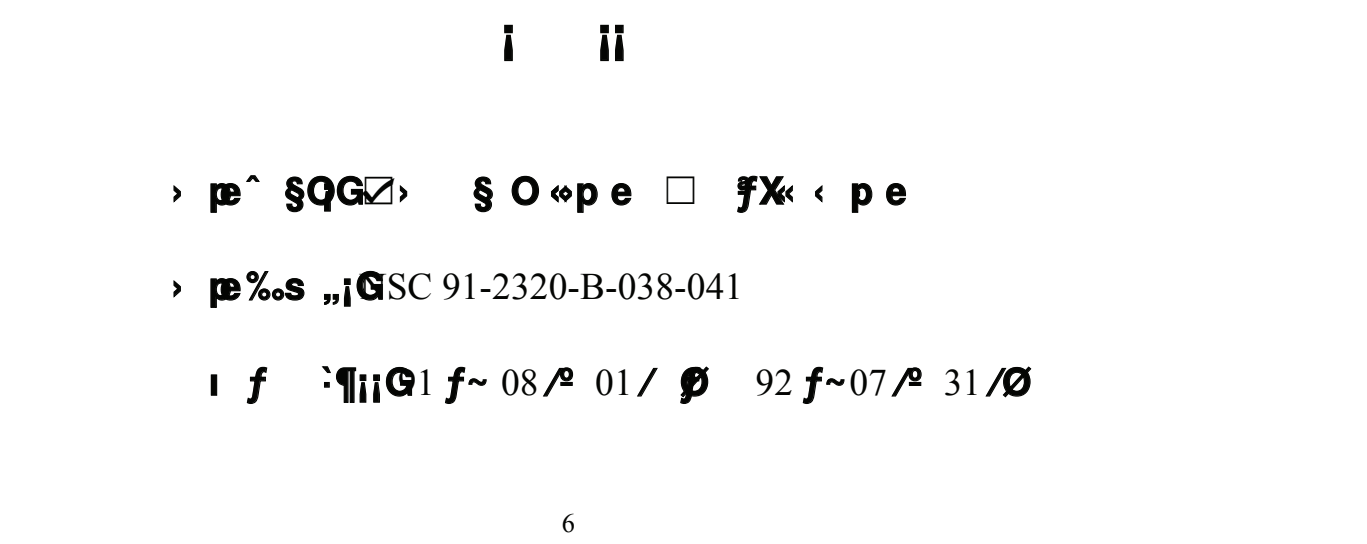

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