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Analytical Biochemistry 319 (2003) 34–41

ANALYTICAL BIOCHEMISTRY

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Identification of L-3-hydroxybutyrate as an original ketone body in rat serum by column-switching high-performance liquid chromatography and fluorescence derivatization

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Received 31 December 2002

Abstract

L-3-Hydroxybutyrate (L-3HB), the enantiomer of D-3-hydroxybutyrate (D-3HB), has traditionally been regarded the "unnatural" ketone body in mammals, although there is suspicion that it is a more-favorable energy fuel for mammalian tissues than D-3HB. In this study, we demonstrated that L-3HB is an original substance in rat serum by applying fluorescence derivatization and a column-switching high-performance liquid chromatography system as the analysis technique. Racemic 3HB in rat serum derivatized using 4-nitro-7-piperazino-2,1,3-benzoxadiazole 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. 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 μM (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

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,[1] 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 and 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 metabolized not 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

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[1] *Abbreviations used:* D-3HB, D-3-hydroxybutyrate; L-3HB, L-3-hydroxybutyrate; DBD-PZ, 4-(*N*,*N*-dimethyl-aminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole; NBD-PZ, 4-nitro-7-piperazino-2,1,3-benzoxadiazole; TPP, triphenylphosphine; DPDS, 2,2′-dipyridyl disulfide; TFA, trifluoroacetic acid; MeCN, acetonitrile; EtOH, ethanol; MeOH, methanol; I.S., internal standard.

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doi:10.1016/S0003-2697(03)00283-5

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; 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 fluorometric 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 precolumn derivatization is described.

Materials and methods

Chemicals

Sodium D- and L-3HB were purchased from Wako Pure Chemicals (Osaka, Japan). DBD-PZ, NBD-PZ, triphenylphosphine (TPP), and 2,2′-dipyridyl disulfide (DPDS) were from Tokyo Kasei Kogyo (Tokyo, Japan). Trifluoroacetic acid (TFA) was purchased from Riedel-de Haën (Seelze, Germany), and propionic acid was from Nacalai Tesque (Kyoto, Japan). Tris(hydroxymethyl)aminomethane (Tris), NAD, and D-3HB dehydrogenase were obtained from Sigma (St. Louis, MO, USA), and perchloric acid was from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile (MeCN), ethanol (EtOH), and methanol (MeOH) were from Merck (Darmstadt, Germany). TSKgel ODS-80Ts (150×4.6 mm i.d.) and Ultron ES-PhCD (phCD) (150×6.0 mm i.d.) were from the Tosoh Co. (Tokyo, Japan) and Shinwa Chemical Industries (Kyoto, Japan), respectively. CHIRALPAK AD-RH (AD-RH) (150×4.6 mm i.d.) and CHIRALCEL OD-RH (OD-RH) (150×4.6 mm i.d.) are the products of the Daicel Co. (Osaka, Japan).

Derivatization of D- and L-3HB with DBD-PZ or NBD-PZ

D- and L-3HB derivatized with DBD-PZ or NBD-PZ were prepared via the principle of the derivative reaction as shown in Fig. 1. In trials in which we were looking for adequate enantiomeric separation, 50 μl of 0.50 mM sodium racemic 3HB (D:L = 1:1) in H_2O was used and brought to 200 μl with EtOH. One hundred microliters of the racemic 3HB solution was added to 100 μl of 2 mM DBD-PZ or NBD-PZ dissolved in MeCN, and then 50 μl each of 280 mM TPP and DPDS in MeCN was added and mixed. The derivatization was allowed to stand for 3 h at 30 °C, followed by addition of 250 μl of 0.1% TFA in H_2O to terminate the reaction. An Empore SDB-RPS cartridge (3M, St. Paul, MN, USA) was used for removing the excess DBD-PZ or NBD-PZ in the resultant solution, as these fluorogenic reagents were protonated in the acidic condition and would be retained on the cartridge via higher cation exchange affinity than that of derivatized products. The cartridge was preconditioned with 100 μl of an eluting solution composed of EtOH/MeCN/H_2O (20/30/50, v/v/v), and the eluent was discarded. We then loaded 100 μl of the resultant solution onto the cartridge and collected the eluent. Another elution was performed by loading 100 μl of the eluting solution to thoroughly elute D- and L-3HB. Two portions of the eluent were combined and filtrated through 0.20-μm filters (Sartorius AG, Göttingen, Germany), and 20 μl of the filtrate was injected into the HPLC.

Preparation of rat serum

Serum used for L-3HB analysis was from 6-week-old Sprague–Dawley rats (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan), which

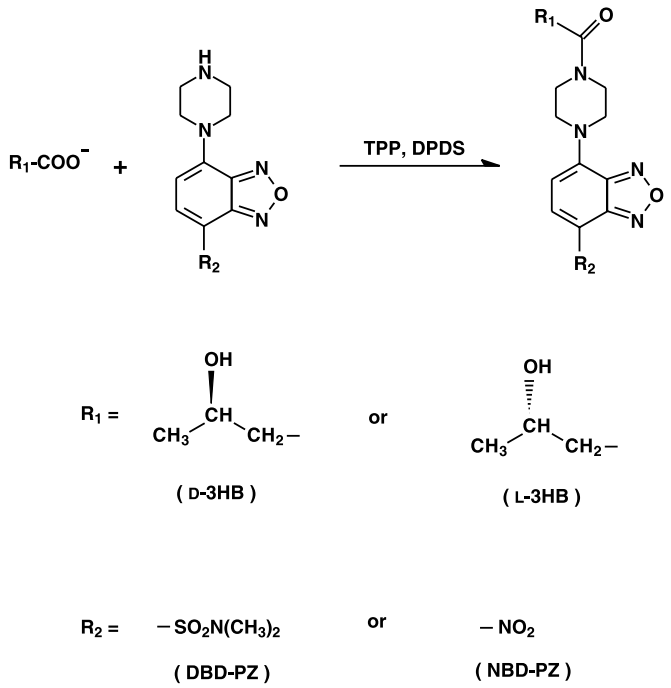

Fig. 1. Derivatization scheme of D- and L-3HB with DBD-PZ or NBD-PZ. TPP and DPDS were used as the condensing agents.

were reared in cages with food and tap water provided ad libitum. To obtain serum, blood samples drawn from the tail vein were centrifuged at 3300*g* for 15 min at 4 °C. Ten microliters of 1.0 mM propionic acid in H_2O as an internal standard (I.S.) was added to 50 µl of serum and then brought to 200 µl with EtOH for deproteinization. The solution was vigorously mixed using a vortex mixer and centrifuged at 700*g* for 5 min. One hundred microliters of the supernatant was supplemented with fluorogenic reagents and TPP and DPDS to perform the derivatization as described above.

HPLC conditions

A column-switching HPLC system, as described in previous publications [14,15], was used in the present work. As illustrated in Fig. 2, the HPLC system was equipped with a Rheodyne Model 7725i sample injector with a 20-µl loop, two pumps (L-7100; Hitachi, Tokyo, Japan), an F-1000 and an L-7485 fluorescence detector (Hitachi), two D-2500 chromatointegrators (Hitachi), and a Rheodyne Model 7000 switching valve with a 100-µl loop as a sample trap.

A TSKgel ODS-80Ts column was used for isolation and quantification of the racemic 3HB in rat serum; the mobile phase was MeOH/H_2O (33/67, v/v) at a flow rate of 0.7 ml/min. Several chiral columns including AD-RH, OD-RH, phCD, or two OD-RHs connected in tandem (tandem OD-RHs) were used for the enantiomeric separation of D- and L-3HB eluted with either MeOH/H_2O (80/20, v/v) or MeCN/H_2O (40/60, v/v); the flow rate was isocratic at 0.3 ml/min. Fluorescence detection (detectors 1 and 2) was performed at 560 nm with a 450-nm excitation wavelength or at 547 nm with a 491-nm excitation wavelength for DBD-PZ and NBD-PZ, respectively.

As the monitored peak top of racemic 3HB appeared on the chromatograms in Integrator 1, a portion of eluted racemic 3HB was presumed to be isolated in the sample trap, and the valve position was manually switched from A (solid line) to B (dotted line) to introduce

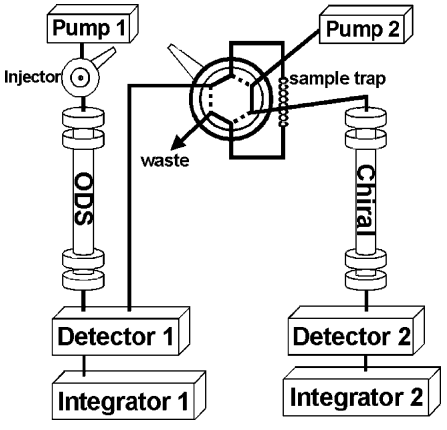

Fig. 2. HPLC system with a column-switching valve used in this study. The six-port valve positions A and B are represented by the solid and dotted lines, respectively. A TSKgel ODS-80Ts column was used as the ODS column, and AD-RH, OD-RH, phCD, and tandem OD-RHs were used for the chiral columns. The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

the racemic 3HB into the chiral column. Then, the D and L isomers were separated enantiomerically on the chiral column, and enantioseparation chromatograms were obtained from Integrator 2. Meanwhile the elution of derivatized rat serum sample by the TSKgel ODS-80Ts column continued and Integrator 1 did not stop recording until the appearance of the I.S. eluted at about 43 min. The valve position was switched back to position A before the next injection.

Calibration curve

For construction of the calibration curve, 50 μl of racemic 3HB (D:L = 1:1) at concentrations of 0.05, 0.10, 0.20, 0.30, 0.40, and 0.50 mM was added with 10 μl of 1.00 mM propionic acid in H_2O (I.S.) and then brought to 200 μl with EtOH. Derivatization procedures were carried out in the same manner as described above ($n = 3$). The calibration curve was plotted from the peak area ratio of racemic 3HB to that of I.S. vs the concentrations of racemic 3HB.

Confirmation of racemic 3HB with D-3HB dehydrogenase

Utilization of D-3HB dehydrogenase for the conversion of D-3HB to acetoacetate was a modification of the method proposed by Ruell and Gass [16]. Briefly, 0.75 ml of rat serum was deproteinized with 1.5 ml of 0.6 M ice-cold perchloric acid. After shaking vigorously and centrifuging at 3000*g* for 15 min, the supernatant was transferred to an Eppendorf tube as the sample for the oxidation by D-3HB dehydrogenase.

A solution of 1.5 M Tris and NAD was prepared at a ratio of 1 mg NAD to 0.7 ml Tris. Tris/NAD (0.7 ml) was supplemented with 0.3 ml of sample and 10 μl of D-3HB dehydrogenase (5 mg/ml). This mixture was allowed to stand for 1 h at 37 °C. As a control, D-3HB dehydrogenase was replaced with 10 μl H_2O under the same conditions. Subsequently, 50 μl of the mixture was brought to 200 μl with EtOH to perform derivatization and analysis on HPLC.

Validation study

To estimate the precision and accuracy of the present analytical method, intra- and interday determinations of L-3HB in rat serum were carried out by adding different concentrations of sodium L-3HB (0, 2.00, 4.00, and 6.00 μM) to 50 μl of rat serum with 10 μl of 1.0 mM propionic acid (I.S.). Each sample was analyzed by the proposed method as described above ($n = 5$). The precision is presented as the relative standard deviation (RSD), and the accuracy is expressed as the calculated recovery.

Results and discussion

Enantiomeric separation of D- and L-3HB by chiral columns

The chiral columns including AD-RH, OD-RH, phCD, and tandem OD-RHs were used with various mobile phase compositions to try to develop an adequate separation method. The compositions of MeOH/H_2O (80/20) and MeCN/H_2O (40/60) were found to elute better enantioseparation over all tested conditions, and the resultant k', α, and Rs values are listed in Table 1.

When D- and L-3HB were derivatized with NBD-PZ, the use of polysaccharide-based chiral columns (AD-RH, OD-RH, and tandem OD-RHs) could obtain higher α and Rs values than a phCD column, and the same results held with both mobile phase compositions. However, polysaccharide-based columns produced no separation on D- and L-3HB derivatized with DBD-PZ, and using a phCD column achieved Rs values of 1.13

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			
	$k'_{\text{L-3HB}}$	$k'_{\text{D-3HB}}$	α	Rs	$k'_{\text{L-3HB}}$	$k'_{\text{D-3HB}}$	α	Rs
MeOH/H_2O (80/20)								
AD-RH	9.91	14.25	1.44	1.98	5.25	5.25	1	—
phCD	4.26	4.65	1.09	1.00	1.55	1.72	1.11	1.13
OD-RH	5.45	7.38	1.35	3.48	2.83	2.83	1	—
Tandem OD-RHs	5.78	7.59	1.31	3.30	3.13	3.13	1	—
MeOH/H_2O (40/60)								
AD-RH	2.65	2.94	1.11	0.77	1.32	1.32	1	—
PhCD	2.07	2.20	1.06	0.73	1.63	1.75	1.07	0.80
OD-RH	1.35	1.47	1.09	1.12	1.23	1.23	1	—
Tandem OD-RHs	1.50	1.63	1.09	1.60	1.31	1.31	1	—

and 0.80 with MeOH/H_2O (80/20) and MeCN/H_2O (40/60), respectively. Under all trials, the L configurations were eluted before the D configurations.

The NBD-PZ derivatives were found to be retained on the phCD column to a greater extent than the DBD-PZ derivatives, suggesting that a stronger hydrophobic interaction was occurring between the NBD-PZ derivatives and the phenylcarbomylated moieties within the β-cyclodextrin cavities. However, broad peak shapes were also exhibited on the chromatograms at the same time, which are detrimental to quantification of low concentrations of L-3HB. The best enantiomeric performance with the use of phCD occurred with DBD-PZ derivatives eluted with the mobile phase of MeOH/H_2O (80/20); yet this condition was unsatisfactory for the present research. The main reason for the inefficient resolution was proposed to be the difficulty in penetrating the β-cyclodextrin cavity by the bulky structure of the NBD-PZ or DBD-PZ derivatives [17]. Thus, another type of chiral column, the polysaccharide-based stationary phase, was subsequently investigated.

Generally, the interactions of the targeted racemate retained on the polysaccharide-based stationary phases, including cellulose tris(3,5-dimethylphenyl carbamate) on OD-RH or amylose tris(3,5-dimethylphenyl carbamate) on AD-RH, occurred via hydrogen bonding, dipole–dipole interactions with the carbamoyl groups, and π–π interactions between the 3,5-dimethylphenyl ring and the aromatic groups of the racemate [18]. In the work of Guo et al. [15], the fluorogenic reagents NBD-COCl and DBD-COCl, whose chemical structures are similar to NBD-PZ and DBD-PZ, were used to react with racemic fluoxetine to perform enantiomeric separation on AD-RH or OD-RH columns. Higher values of Rs and α were obtained with derivatives of NBD-COCl than with those of DBD-COCl. Therefore, the superior resolution of NBD-PZ derivatives compared to that of DBD-PZ derivatives may have been due to the same mechanism observed by Guo and co-workers, who suggested that the NBD moiety is a more-intense π acceptor than the DBD moiety, so that stronger π–π interactions occur between NBD derivatives and phenyl groups on the stationary phase. Hence, NBD-PZ was thereafter selected as the fluorogenic reagent in the present study.

Although D- and L-3HB derivatized with NBD-PZ could be completely separated on both AD-RH and OD-RH columns with the mobile phase of MeOH/H_2O (80/20), the retention times of D- and L-3HB derivatives dramatically increased. With regard to the D-3HB derivative, the last eluted isomer, the retention time was prolonged from 25 to 97 min for AD-RH and from 16 to 54 min for OD-RH, as compared to the use of the mobile phase of MeCN/H_2O (40/60). In addition, the peaks were very broad, and tailing occurred. It seemed that the enantiomeric resolution of NBD-PZ derivatives was enhanced by replacing MeCN with MeOH, but this would be contradictory when considering interactions on the NH group of the carbamate moiety of AD-RH or OD-RH columns. Electronegative atoms such as the oxygens of NBD-PZ derivatives are capable of interacting with NH groups through hydrogen bonding [19], and so is MeOH [17]. Competition between MeOH and NBD-PZ derivatives would thus result in poor enantiomeric resolution, as is the case of enantiomeric separation of β blockers derivatized with DBD-COCl on cellulose chiral columns [20]. However, the prolonged retention times and better resolutions in our study when using MeOH/H_2O produced the opposite result, and the mechanism of this conflicting result is still not known.

The three-dimensional structure of the amylose-based chiral column is more helical in nature, has well-defined cavities [21], and is supposed to have stronger interactions with NBD-PZ derivatives than those which occur with a cellulose-based chiral column, as judged from the higher k' values and broader peaks on chromatograms with AD-RH. Despite this, an efficient enantiomeric separation was still not achieved when using AD-RH with the MeCN/H_2O (40/60) mobile phase. Actually the Rs value was higher on the OD-RH than on the AD-RH column; they were 1.12 and 0.77, respectively. It was suggested that the magnitude of interactions between D- and L-3HB derivatives and the amylose tris(3,5-dimethylphenyl carbamate) moieties of AD-RH do not efficiently differ from each other, as interactions occurred on both D and L isomers which only resulted in broader peaks on the chromatograms.

A phenomenon noticed in our laboratory was that the use of AD-RH and MeCN/H_2O as the mobile phase was completely capable of separating D- and L-lactate derivatized with NBD-PZ (unpublished observation). Chemically, D- and L-lactate have just one less $–CH_2–$ group in the alkyl chain than D- and L-3HB. The inefficient resolution of D- and L-3HB derivatives using AD-RH was beyond our expectations, and this result might have been due to the poor interactions caused by repulsion between the longer alkyl chains of D- and L-3HB derivatives and the alkyl chains inside the AD-RH column. Another possible explanation was the lower rigidity of D- and L-3HB, since conformational rigidity has been reported to be important for resolution under reversed-phase conditions [22].

While failing to achieve ideal enantiomeric resolution, chromatograms obtained from OD-RH showed sharp peaks and shorter periods of time required for eluting the D- and L-3HB derivatives. To utilize these favorable characteristics, we connected two OD-RH columns to the HPLC system in tandem. Fig. 3 shows the chromatograms of D- and L-3HB on tandem OD-RH columns using MeCN/H_2O (40/60) as the mobile phase. D- and L-3HB were satisfactorily separated from each other with an Rs of 1.60 in this system, and separation

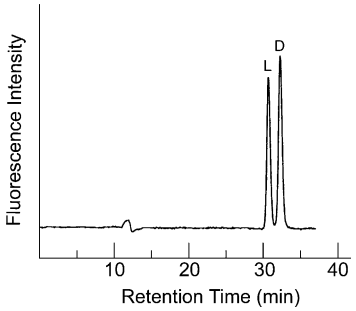

Fig. 3. Enantiomeric separation of standard D- and L-3HB derivatized with NBD-PZ by two OD-RHs connected in tandem. As indicated, the L-3HB derivative was eluted before D-3HB, and the Rs value was 1.60.

could be completed within 40 min. Therefore, we decided to employ two OD-RH columns connected in tandem in the HPLC system for the study of the enantiomeric separation of D- and L-3HB.

Applications to rat serum analysis

Before applying the proposed enantiomeric method to determine L-3HB in rat serum, total racemic 3HB including D and L isomers first had to be isolated. After derivatization, the racemic 3HB could be isolated from other components containing carboxyls in rat serum using a TSKgel ODS column with isocratic elution of MeOH/H_2O (33/67) at a flow rate of 0.7 ml/min. The racemic 3HB peak on the chromatogram eluted at about 26 min, and the retention time of the internal standard was about 43 min (Fig. 4). Neither the racemic 3HB nor the internal standard peaks were found to overlap with any other interfering peaks, and quantification of the racemic 3HB concentration was accomplished through the calibration curve, whose preparation was described under Materials and methods.

In addition to comparing the retention times of standard D- and L-3HB derivatives, D-3HB dehydrogenase was used to further confirm the peak that represented racemic 3HB. This was based on the assumption that the peak area of racemic 3HB should be reduced or disappear when rat serum was pretreated with D-3HB dehydrogenase to convert D-3HB to acetoacetate in advance of derivatization. Since it is known [2] that D-3HB comprises a major portion of the total racemic 3HB (more than 95%), and enzymes that are specific for L-3HB remain to be identified, using D-3HB dehydrogenase became a better choice for verifying racemic 3HB on the chromatograms. As expected, Fig. 5 shows the chromatograms of rat serum with or without pretreatment with D-3HB dehydrogenase, and the proposed racemic 3HB peak vanished in the presence of D-3HB dehydrogenase. An increased peak area eluted at 22 min was found on the chromatogram (Fig. 5B), and this was supposed to be the mixtures of metabolites such as acetoacetate or acetic acid after D-3HB dehydrogenase

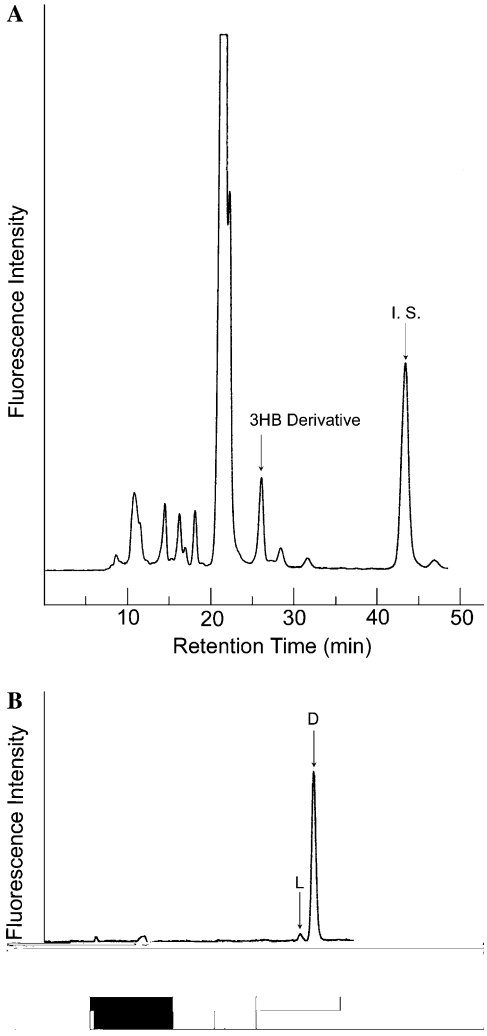

Fig. 4. Simultaneous analysis of L-3HB in rat serum. (A) Rat serum derivatized with NBD-PZ was eluted by the TSKgel ODS-80Ts column and the chromatogram was obtained from Integrator 1. The retention times of the total racemic 3HB derivative and the I.S. were about 26 and 43 min, respectively. (B) Enantiomeric 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.

catabolism. Due to the extra process of addition of perchloric acid for deproteinization and D-3HB dehydrogenase with NAD for converting D-3HB to acetoacetate, the concentration of racemic 3HB in rat serum was even more diluted than that in the previously described analytical method. Therefore the racemic 3HB peak was rather small compared to the chromatograms in Fig. 4A.

The calibration curve prepared from standard D- and L-3HB revealed a linear relationship between the concentrations and the peak response, with a correlation coefficient of 0.998. Accordingly, the concentration of racemic 3HB in normal rat serum could be calculated to be an average of 110.17 μM.

With regard to the simultaneous determination ofLL

These results indicate that the present method was validated.

In conclusion, employing tandem OD-RHs chiral columns enables efficient enantiomeric separation of D- and L-3HB, and with a previous isolation of racemic 3HB by the ODS column, we were able to detect and identify L-3HB in rat serum. Since the D:L ratio of 3HB has been suggested to imply altered selectivity and activity of enzymes responsible for 3HB, use of the method described in this paper to estimate D- and L-3HB variation under physiological and pathological conditions will become practical.

Acknowledgments

This work was financially supported by the National Science Council of the Republic of China (NSC 90-2320-B-038-029). The authors express their sincere appreciation to Professor K. Imai and Dr. T. Fukushima (Tokyo University, Tokyo, Japan) for their helpful suggestions.

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