Clinica Chimica Acta 429 (2014) 90–95

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

HPLC determination of D-3-hydroxybutyric acid by derivatization with a benzofurazan reagent and fluorescent detection: Application in the analysis of human plasma

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ARTICLE INFO

Article history:
Received 2 October 2013
Received in revised form 14 November 2013
Accepted 26 November 2013
Available online 4 December 2013

Keywords:
Biological samples
Clinical/biomedical analysis
Fluorescence/luminescence
HPLC

ABSTRACT

A simple and sensitive new method for the determination of D-3-hydroxybutyric acid (D-3-HBA) in human plasma after derivatization is described. The proposed method is based on the reaction of (2*S*)-2-amino-3-methyl-1-[4-(7-nitro-benzo-2,1,3-oxadiazol-4-yl)-piperazin-1-yl]-butan-1-one (NBD-PZ-Val) with D-3-HBA in the presence of *O*-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and *N*-ethyldiisopropylamine (DIEA) to produce a fluorescent derivative. The formed derivative was monitored fluorimetrically at $\lambda_{ex} = 489$ nm and $\lambda_{em} = 532$ nm. The HPLC analysis was carried out by use of a C18 analytical column (Synergy Hydro 150 mm × 3 mm, i.d., 4 μm) with a binary gradient elution program of 0.1% aqueous trifluoroacetic acid versus methanol. The method showed satisfactory linearity ($r^2 = 0.9997$) in the range from 20 to 500 μmol/L. The limit of detection (LOD) of the method was 7.7 μmol/L, while the limit of quantitation (LOQ) was 25.8 μmol/L. The analytical method was successfully applied to human plasma samples from normal healthy subjects.

1. Introduction

Serum concentration of D-3-hydroxybutyric acid (D-3-HBA) is an indicator of the status of hepatic fatty acid oxidation that, when enhanced results in the increased production of acetoacetic acid which is converted into D-3-HBA by a reaction catalyzed by *D-3-hydroxybutyrate dehydrogenase*. D-3-Hydroxybutyric acid represents a major form of ketone bodies, especially in such metabolic situations as starvation [1,2] and diabetes mellitus [2], by which hepatic fatty acid oxidation is enhanced.

For this purpose, several enzymatic assays for the determination of this compound have been reported [3–9]. The principle of these assays is based on the enzymatic method of Williamson and coworkers [3], where the bidirectional reaction of *D-3-hydroxybutyrate dehydrogenase* is used for reversibly converting D-3-hydroxybutyrate to acetoacetate and the increase or decrease in absorbance at 340 nm is monitored [3–5]. The enzyme-spectrophotometric method specifically determines D-3-HBA and is simple and accurate; however this method is not sensitive enough and, therefore, it requires a large volume of the serum sample to obtain a reliable value.

Subsequently Yamato et al. developed a spectrophotometric method for the differential determination of ketone bodies employing *p*-nitrobenzene diazonium fluoroborate (diazo reagent) as a color-developing reagent [10]. Unfortunately, the diazo reagent lacks the required specificity for certain clinical applications. Diazo reagent can react with compounds such as oxaloacetate, antidiabetes mellitus drugs and other drugs in current medical usage.

More recently, a method for analyzing D-3-HBA by gas chromatography–mass spectrometry (GC–MS) has been reported [11] that is applicable to blood and urine samples containing this compound at a concentration higher than 20 μmol/L. However, this method has only a moderate sensitivity and, furthermore, it requires specific and expensive instrumentation.

Following a different approach, Tsai et al. [12] and Hsu et al. [13] have developed a pre-column fluorescence derivatization method for the enantioselective determination of both enantiomers of 3-hydroxybutyrate in the tissues of normal and diabetic rats, employing a column-switching HPLC system.

Given these limitations and on the basis of our previous experience in this field [14], we have developed and validated a sensitive and convenient high-performance liquid chromatography (HPLC) method for the quantitative determination of the D enantiomer of 3-hydroxybutyric acid.

Although its significance from a clinical standpoint is lower, L-3-hydroxybutyric acid is also present in biological fluids; thus, for the

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http://dx.doi.org/10.1016/j.cca.2013.11.030

determination of the D enantiomer in serum, separation of the two individual enantiomers is required.

Two strategies have mainly evolved for the separation of a pair of enantiomers by HPLC. The first one is the "direct method" using a chiral stationary phase (CSP) [15]. The second one is the "indirect technique" based upon diastereomer formation with a suitable chiral derivatization reagent [16–24]. This indirect method involving a derivatization step is suitable for the trace analysis of enantiomers in biological samples, such as blood and urine, because a highly sensitive detection can be performed with the option of coupling analytes with suitable chiral reagents which have a high molar UV–Vis absorptivity (ε), or high fluorescence (FL) quantum yield (Φ). The labeling of chiral compounds with a reagent absorbing in the UV or Vis region is the most popular means of derivatization. Therefore, many UV labels have been applied to the tagging of various functional groups. However, in some real samples the absorptivity of the so-obtained derivatives is not high enough to ensure an adequate sensitivity. Very recently, a sensitive method for the detection of trace amounts of enantiomers of lactic and 3-hydroxybutyric acids in human saliva has been reported [24]. However, the employed derivatization reaction is quite slow, 90 min, and the method requires the use of UPLC–MS/MS technique, a quite expensive instrumentation often out of reach for a number of laboratories.

Thus, a further selective and sensitive detection system is required for the analysis of small quantities of chiral compounds in complex matrices such as biological samples. The FL labeling is one of the most effective techniques for quantitative determinations in biological specimens, in terms of sensitivity and/or selectivity.

We have previously synthesized a new chiral, fluorescent derivatization reagent, having the benzofurazan structure, (2*S*)-2-amino-3-methyl-1-[4-(7-nitro-benzo-2,1,3-oxadiazol-4-yl)-piperazin-1-yl]-butan-1-one (NBD-PZ-Val), which reacts easily and quantitatively with D- and L-lactic acid to form the corresponding stable, highly fluorescent diastereomeric amides [14]. In this paper, we describe a highly sensitive RP-HPLC method for the determination of D-3-HBA in human plasma with fluorescence detection after pre-column derivatization with NBD-PZ-Val (Fig. 1) in the presence of a coupling agent (HATU/DIEA).

2. Experimental section

2.1. Chemicals

4-Nitro-7-piperazinobenzofurazan (NBD-PZ) and *N*-ethyldiisopropylamine (DIEA) were obtained from Fluka; *O*-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from Merck (Germany); D,L-3-hydroxybutyric and D-3-hydroxybutyric acid sodium salts, (*S*)-2-(Boc-amino)-3-methylbutyric acid (Boc-L-valine; enantiomeric purity ≥99.0%), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF) and hydrochloric acid were from Sigma–Aldrich. NBD-PZ-Val was synthesized in our laboratory [14]. All other reagents were of the highest purity available.

2.2. Plasma samples

Plasma samples were obtained through a standardized procedure. Human whole blood was collected into tubes containing EDTA as an anticoagulant. Plasma was obtained immediately by the centrifugation of the blood at 2000 *g* for 15 min, and samples were stored at −80°C until analysis.

2.3. Sample preparation

Forty microliters of sample (either human plasma or 20–500 μmol/L solutions of sodium D-3-hydroxybutyrate or D,L-3-hydroxybutyrate in water) was mixed with 40 μL of water and with 120 μL of the deproteinizing solvent CH_3CN, and the mixture centrifuged for 6 min at 14,500 ×*g*. Following this, 40 μL of the resulting solution were added to 40 μL of 6 mmol/L NBD-PZ-Val in CH_3CN in the presence of 40 μL of HATU and DIEA (both 8 mmol/L in CH_3CN), and the resulting solution was incubated at 30 °C for 5 min, then acidified with 80 μL of 1 mol/L HCl.

2.4. Apparatus and chromatographic conditions

HPLC analyses were carried out on a Agilent 1100 system (Agilent Technologies, Inc.) equipped with an online vacuum degasser, a high-pressure gradient quaternary pump, a manual sample injector (loop 5 μL), a column oven and a fluorescence detector. Data analyses were performed using Agilent ChemStation software (Agilent Technologies).

The HPLC separations of the derivatized racemic 3-HBA and D-3-HBA were performed on a reversed phase C18 column Synergy Hydro (Phenomenex, Torrance, CA, USA) 150 mm × 3 mm (i.d.) with 4 μm particle size, protected by a Phenomenex C18 security guard column (4.0 mm × 3.0 mm). 0.1% aqueous trifluoroacetic acid and MeOH were the mobile phases A and B, respectively. The separation was carried out at a flow rate of 600 μL/min, equilibrating the column at 30% B; the gradient increased linearly up to 50% of B in 20 min, then it reached 100% of B in 5 min and was further maintained at 100% B for 2 min for washing the column. Re-equilibration was carried out over 8 min; total analysis time was 35 min. The column heater was set at 30 °C. Typically, 5 μL of sample was injected onto the column. Derivatized D-3-HBA was detected by fluorescence with excitation at 489 nm and emission at 532 nm.

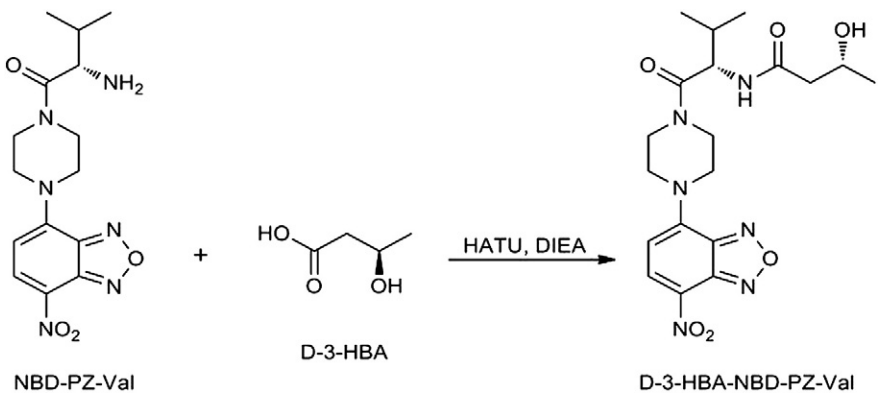

Fig. 1. Derivatization of D-3-hydroxybutyric acid by means of NBD-PZ-Val to afford D-3-HBA-NBD-PZ-Val.