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High-performance liquid chromatography determination of ketone bodies in human plasma by precolumn derivatization with *p*-nitrobenzene diazonium fluoroborate

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ABSTRACT

We developed and validated a sensitive and convenient high-performance liquid chromatography (HPLC) method for the specific determination of ketone bodies (acetoacetate and D-3-hydroxybutyrate) in human plasma. *p*-Nitrobenzene diazonium fluoroborate (diazo reagent) was used as a precolumn derivatization agent, and 3-(2-hydroxyphenyl) propionic acid was used as an internal standard. After the reaction, excess diazo reagent and plasma proteins were removed by passing through a solid-phase cartridge (C_{18}). The derivatives retained on the cartridge were eluted with methanol, introduced into the HPLC system, and then detected with UV at 380 nm. A calibration curve for acetoacetate standard solution with a 20-μl injection volume showed good linearity in the range of 1 to 400 μM with a 0.9997 correlation coefficient. For the determination of D-3-hydroxybutyrate, it was converted to acetoacetate before reaction with the diazo reagent by an enzymatic coupling method using D-3-hydroxybutyrate dehydrogenase and lactate dehydrogenase. A calibration curve for D-3-hydroxybutyrate standard solution also showed good linearity in the range of 1.5 to 2000 μM with a 0.9988 correlation coefficient. Analytical recoveries of acetoacetate and D-3-hydroxybutyrate in human plasma were satisfactory. The method was successfully applied to samples from diabetic patients, and results were consistent with those obtained using the thio-NAD enzymatic cycling method used in clinical laboratories.

Insulin deficiency with glucagon excess leads to increased β-oxidation of free fatty acids. Moreover, insulin imbalance results in the production of ketone bodies (acetoacetate, 3-hydroxybutyrate, and acetone) in the liver that later appear in the blood and urine. Acetoacetate and D-3-hydroxybutyrate, but not volatile acetone, in the blood are clinically used as markers for diagnosing type 1 (insulin-dependent) diabetes [1–3]. Diabetic ketoacidosis is an acute and life-threatening metabolic complication of diabetes that is associated with elevated levels of ketone bodies in the blood and metabolic acidosis. Frequent blood ketone monitoring is efficient for sick day management of type 1 diabetes, and it can prevent or reduce the occurrence and severity of diabetic ketoacidosis [4]. In addition, blood ketone monitoring is useful for providing nutritional recommendations to women with gestational diabetes [5]. Ozawa demonstrated that the ketone body ratio (acetoacetate/D-3-hydroxybutyrate) in arterial blood reflected the mitochondrial redox state in liver tissue [6]. Subsequent research has reported the ketone body ratio in the blood to be a useful index for assessing hepatic functional reserve after hepatectomy [7], a prognostic indicator in acute heart failure [8], and a stress marker in acute myocardial infarction [9].

A number of enzymatic assays for the determination of ketone bodies have been reported [10–15]. The principle of these assays is based on the enzymatic method of Williamson and coworkers [10], where the bidirectional reaction of D-3-hydroxybutyrate dehydrogenase is used for reversibly converting D-3-hydroxybutyrate to acetoacetate and the increases or decreases in absorbance at 340 nm are monitored [10–12]. Aside from this approach, production of the reduced form of nicotinamide adenine dinucleotide (NADH)[1] has also been coupled with the reaction of NADH oxidase, followed by color

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[1] *Abbreviations used:* NADH, nicotinamide adenine dinucleotide, reduced form; NAD^+, nicotinamide adenine dinucleotide, oxidized form; HPLC, high-performance liquid chromatography; IS, internal standard; β-NAD, β-nicotinamide adenine dinucleotide; LOD, limit of detection; LOQ, limit of quantitation; QC, quality control; RSD, relative standard deviation.

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development of chromogen with hydrogen peroxide produced by the oxidase reaction; here the amount of oxidized chromogen produced, which is proportional to D-3-hydroxybutyrate concentration, is measured [13]. In the case of a handheld ketone sensor, the produced NADH is reoxidized to NAD^+ by a redox mediator, and the generated electric current, which is proportional to the blood D-3-hydroxybutyrate concentration, is directly measured [14]. The cyclic thio-NADH method has also been developed for the determination of ketone bodies [15]. Here the reversible reaction of D-3-hydroxybutyrate dehydrogenase accumulates thio-NADH, and this increases the absorbance at 405 nm. Overall, enzymatic methods are simple, specific, and sensitive, and they can be incorporated into an automated analyzer. However, these methods are not a good approach for detecting ketone bodies directly because their detection concept is based on the principle that changes in NADH concentration is proportional to ketone body concentration. Therefore, contamination of commercial D-3-hydroxybutyrate dehydrogenase with other oxidoreductases (e.g., lactate dehydrogenase, malate dehydrogenase) or the presence of endogenous oxidoreductases in plasma samples interferes with the accurate determination of ketone bodies. To this end, chemical approaches for directly detecting acetoacetate in the blood have been reported [16–18]. In these approaches, acetoacetate is coupled with benzene diazonium salts to form intensely colored products. Harano and coworkers used *p*-nitrobenzene diazonium fluoroborate (diazo reagent) as a color-developing reagent for measuring acetoacetate concentration in serum spectrophotometrically [18]. This reagent reacts with activated methylene and at the *o*- or *p*-position of phenolic compounds and, therefore, lacks the required specificity for certain clinical applications. We have applied the diazo reagent as a postcolumn derivatization tool for the specific determination of acetoacetate in high-performance liquid chromatography (HPLC) and used it for the accurate determination of ketone bodies in serum and urine without any interference by oxaloacetate, especially in urine, and by other interference compounds [19]. However, this previous method is not sensitive enough to determine low concentrations of ketone bodies and requires procedures of deproteinization and centrifugation.

In this article, we describe a sensitive and convenient HPLC method in which the diazo reagent was used as a precolumn derivatization agent, and 3-(2-hydroxyphenyl) propionic acid was used as an internal standard (IS), for the specific determination of ketone bodies in human plasma. We employed this precolumn HPLC method to specifically determine ketone bodies in plasma of healthy volunteers and patients with diabetes mellitus. This new method was also compared with the thio-NAD enzymatic cycling method.

Materials and methods

Reagents

Lithium acetoacetate, D-3-hydroxybutyrate sodium salt, β-nicotinamide adenine dinucleotide (β-NAD), D-3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas* spheroids (type 2, suspension in 3.2 M ammonium sulfate solution, pH 6.0, 8.2 U/mg protein), and L-lactate dehydrogenase from rabbit muscle (type 2, suspension in 3.2 M ammonium sulfate solution, pH 6.0, 1150 U/mg protein) were purchased from Sigma (St. Louis, MO, USA). 3-(2-Hydroxyphenyl) propionic acid was obtained from Aldrich (Milwaukee, WI, USA). Sodium pyruvate and polyoxyethylene octylphenylether (Triton X-100) were obtained from Wako (Osaka, Japan). *p*-Nitrobenzene diazonium fluoroborate was purchased from Tokyokasei (Tokyo, Japan). Methanol was of HPLC grade, and all other reagents were of analytical reagent grade.

Preparation of samples

The subjects included 140 consented patients admitted to Niigata University Medical and Dental Hospital who received treatment for diabetes. An additional 30 healthy volunteers also provided written informed consent. Blood samples were collected by venipuncture into an evacuated tube with heparin, centrifuged, and then stored at −80 °C until the assay.

Reaction of diazo reagent with acetoacetate or 3-(2-hydroxyphenyl) propionic acid

p-Nitrobenzene diazonium fluoroborate was dissolved in 0.2% Triton X-100 solution to a final concentration of 10 mM and designated as the diazo reagent solution. Then 100 μl of 0.2 mM acetoacetate or 0.2 mM 3-(2-hydroxyphenyl) propionic acid was added to 100 μl of 0.4 M citrate buffer (pH 3.5), followed by the mixing of 100 μl of 10 mM diazo reagent solution. After incubation at 37 °C, 20-μl aliquots of the reaction mixture were directly introduced to the HPLC system at certain time intervals.

Spectrum measurement

Here 100 μl of 0.2 mM acetoacetate or 3-(2-hydroxyphenyl) propionic acid was added to 100 μl of 0.4 M citrate buffer (pH 3.5), followed by the mixing of 100 μl of 10 mM diazo reagent solution and incubation for a certain time at 37 °C. Thereafter, the mixture was immediately loaded on a Bond Elut C_{18} cartridge (50 mg, 1 ml, Varian Palo Alto, CA, USA) that was preconditioned by washing with 1 ml of methanol and 1 ml of water and then was washed with 3 ml of water to remove excess diazo reagent. Next, each reaction product retained on the cartridge was eluted with 2.4 ml of methanol from the Bond Elut C_{18} cartridge. Absorption spectra of methanol eluates were monitored at wavelengths from 310 to 500 nm with a UV–visible spectrophotometer (Shimadzu UV-2550, Shimadzu, Kyoto, Japan). The methanol eluates obtained after a 2-min reaction were further diluted with 1.6 ml of methanol, 0.4 M citrate buffer (pH 3.5), 0.4 M borate buffer (pH 9.0), or 1 mM hydrochloric acid and then were allowed to stand for some time at room temperature. Absorption spectra of the diluted solutions were also monitored.

Enzymatic conversion of D-3-hydroxybutyrate to acetoacetate

D-3-Hydroxybutyrate was enzymatically converted to acetoacetate prior to reaction with the diazo reagent following the same procedure described previously [19] with slight modification. In brief, 25-μl aliquots of the standard solution or plasma sample containing D-3-hydroxybutyrate were incubated with 50 μl of an enzyme reagent cocktail consisting of 500 μl of 50 mM phosphate buffer (pH 8.0), 100 μl of D-3-hydroxybutyrate dehydrogenase (16 kU/L), 100 μl of L-lactate dehydrogenase (6.7×10^3 kU/L), 100 μl of 10 mM β-NAD, and 100 μl of 0.1 M sodium pyruvate. The reaction mixture was incubated at 37 °C for 10 min, and then 50-μl aliquots of the mixture were used for making the calibration curve of D-3-hydroxybutyrate and also for determining total ketone bodies in plasma.

Determination of ketone bodies in plasma

Determination of acetoacetate was carried out using the IS method. First, 50 μl of 0.2 mM 3-(2-hydroxyphenyl) propionic acid as the IS was added to 100 μl of 0.4 M citrate buffer (pH 3.5), followed by the mixing of 50 μl of 10 mM diazo reagent solution. After incubation for 5 min at 37 °C, 25-μl aliquots of the working standard solution of acetoacetate or the plasma sample were