Critical Role of pH for Derivatization of Homocysteine with Benzofurazanes, Anna Rita Bonfigli,1 Gilberto Coppa,2 Ivoano Testa,3 Anna Gambini,2 and Roberto Testa1 (* Department of Gerontological Research, Diabetology Unit, Italian National Research Centres on Aging, Via Birarelli 8, Ancona, Italy; 2 Department of Clinical Pathology, General Hospital of Ancona, Ancona, Italy; 3 Institute of Internal Medicine, University of Ancona, Ancona, Italy; * author for correspondence: 39-071-2206106, e-mail r.testa@inrca.it)

HPLC analysis combined with fluorescence detection is one of the most effective and widely used techniques for the specific determination of total homocysteine in plasma (tHCY) (1–4). These methods often use benzofurazanes as derivatization reagents that are considered to be specific for the thiol group of HCY (5–14). The specificity of benzofurazanes for thiols has been demonstrated by ABD-F, producing an underestimation of tHCY concentration. Taking into account these findings, we prefer to perform the ABD-F derivatization of tHCY at pH 10.5.

Sample preparation was performed as follows: reduction with tris-carboxyethyl-phosphine, derivatization with ABD-F, and precipitation of proteins. The derivatization was performed before the precipitation to avoid the realkalinization step. To 50 μL of the same sample, we added 100 μL of 250 mmol/L borate buffer at pH 7.5, 8, 8.5, 9, 9.5, 10, and 10.5. We added an internal standard (40 μL of a 50 μmol/L cysteamine solution), and then 30 μL of tris-carboxyethyl-phosphine (9.4 mmol/L in the same borate buffers), followed by 30 μL of ABD-F (9.2 mmol/L in 1 mol/L ammonium hydroxide at the same pH as the borate buffer used). Samples were incubated at 55 °C for 15 min and then cooled at room temperature. Protein was precipitated by adding 50 μL of 600 mL/L perchloric acid. Precipitated HCY-ABD-F derivatives are stable 24 h at room temperature, avoiding direct sunlight.

We injected 50 μL of the supernatant into the chromatographic system [307 Pump HPLC (Gilson), FP 821 Fluorometer (Jasco), HP 3395 Integrator (Hewlett-Packard), and a Genesis octadecysilane C18 column (150 × 4.6 mm i.d.; 4-μm particle size; Jones Chromatography)]. Fluorescence detection used excitation at 385 nm and emission at 515 nm. The mobile phase was 5 mmol/L potassium dihydrogen phosphate buffer containing acetonitrile (30 mL/L) and isopropyl alcohol (30 mL/L), pH 1.9. Recoveries were ~100% (i.e., initial concentration, 9.1 μmol/L; HCY added, 5 μmol/L; within-day recovery, 99.7% ± 2.4%; n = 5), and the assay was linear to 500 μmol/L. Comparison with the fluorescence polarization immunoassay on an IMx analyzer (Abbott) showed the following results: yIMx = 0.878xHPLC + 0.583; r = 0.878; n = 136.

Two chromatograms obtained with the same solution of HCY (200 μmol/L) after derivatization with ABD-F at different pHs are shown in Fig. 1. At pH 10.5, only the −SH ABD derivative peak was present (Fig. 1B), whereas at pH 7.5, two peaks were seen (Fig. 1A), with an −NH2 ABD derivative peak at 6.5 min and the −SH ABD derivative peak at 9.9 min. In Fig. 1E, the percentage of the peak areas of −NH2 ABD derivatives and the −SH ABD derivatives are plotted vs the pH of the ABD-F derivatization. A pH-dependent mirror curvilinear trend is present for both the derivatives.

These data clearly highlight that the pH of derivatization has a critical role in this assay. Small variations of pH can change the results and increase imprecision. Unpredictable variation of plasma amines (e.g., from drugs, illnesses, or diet) may consume ABD-F, producing an underestimation of tHCY concentration. Taking into account these findings, we prefer to perform the ABD-F derivatization of tHCY at pH 10.5.

Chromatograms of the same plasma sample after derivatization at pH 7.5 and 10.5, respectively, are shown in Fig. 1, C and D. Whereas Fig. 1D shows a clean chromatogram with only cysteine, internal standard, cysteinylglycine, and tHCY without other interference, Fig. 1C shows unknown peaks and higher apparent cysteine, presumably reflecting the overlapping of the −NH2 ABD-HCY peak.

We conclude that the pH of the derivatization of tHCY with benzofurazanes must be given particular attention in...
further attempts to standardize HPLC determination of tHcy.

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References


Serum YKL-40 Is Associated with Osteoarthritis and Atherosclerosis in Nonhuman Primates, Thomas C. Register,1* Cathy S. Carlson,2 and Michael R. Adams2 (1 Department of Pathology, Section on Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1040; 2 College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108; * author for correspondence: fax 363-716-1515)

Osteoarthritis (OA) is the leading cause of chronic disability in the US (1). Because individuals with early stages of the disease are often asymptomatic, a biomarker of early OA may be useful. Adult cynomolgus macaques develop naturally occurring OA that closely resembles the human disease (2–4). Radiographic, histologic, and immunohistochemical analyses show a high frequency of lesions in the knee joints of these animals. Lesions are most severe in the medial tibial plateau and are characterized by fibrillation, clefting, and loss of articular cartilage with a concomitant, marked thickening of the subjacent subchondral bone (4).

YKL-40 (also known as human cartilage glycoprotein-39 or 38-kDa heparin-binding glycoprotein), a major secretory protein of human chondrocytes and synovial cells (5), is increased in the serum and synovial fluid of individuals with joint or cartilage disease (5–9). YKL-40 mRNA expression is also increased in the cartilage of patients with rheumatoid arthritis (10). YKL-40, first identified as a major product secreted by osteosarcoma MG63 cells (11), is also produced by fibrotic liver cells and breast cancer cells and not by skin or lung fibroblasts [for a review, see Ref. (8)]. YKL-40 expression is induced during the late stages of differentiation in the transition of monocytes to activated macrophages (12, 13), and YKL-40 mRNA is present in macrophages in human atherosclerotic lesions (14).

The N-terminal amino acid sequence of YKL-40 begins with Tyr-Lys-Leu (YKL), and the protein migrates at 40 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis; hence the YKL-40 designation. YKL-40 binds heparin (15, 16) and chitin and has structural similarity to chitinolytic enzymes, although it lacks chitinase activity (10, 15). Thus, YKL-40 may function as a lectin or glycosyl hydrolase of unknown specificity. YKL-40 expression patterns suggest roles in tissue remodeling or in immune function.

The purpose of the present study was to determine the associations of YKL-40 in serum and synovial fluid with naturally occurring OA and diet-induced atherosclerosis in cynomolgus monkeys. The subjects were subsets of animals obtained from previously described studies [study 1, Adams et al. (17), n = 13; study 2, Williams et al. (18), n = 1] or from a breeding colony (n = 2) at Wake Forest University School of Medicine. Study 1 and 2 animals were ovarioectomized and fed a moderately atherogenic diet (40% of calories as fat and 0.28 mg cholesterol/kcal) for 34 months. Before necropsy, the hips, knees, and feet of the animals were radiographed and sera collected and stored at −70 °C for later analysis. At necropsy, the monkeys were anesthetized deeply with ketamine (250 mg/kg intravenous), and the cardiovascular system was flushed with normal saline. The heart of each animal was excised after ligation of the vena cava, and pulmonary arteries and perfusion were fixed via the aorta with neutral (pH 7.4) phosphate-buffered paraformaldehyde (40 g/L) and sucrose (50 g/L) with EDTA (1 mmol/L) at a pressure of 100 mmHg. The heart was then immersed in the fixative above. Synovial fluid was obtained from the knee joint by injection of 3 mL of sterile saline solution into the joint followed by aspiration, after which the knee joints were collected. Synovial fluid saline aspirates were stored at −70 °C until analysis. All procedures involving animals were conducted in compliance with state and federal laws and standards of the US Department of Health and Human Services and were approved by the Wake Forest University Animal Care and Use Committee.

OA severity in individual animals was ranked as none,