High Performance Liquid Chromatography Combined Identification of Nε-(Carboxymethyl)lysine, Pentosidine, Pyridinoline, and Deoxypyridinoline as Markers of Disease in Musculoskeletal Health

Christian B Allen[1], Cynthia Franklin[2], Jaydee Foster[1], Roberto J Fajardo Ph.D[1], Paulo Carvalho Ph.D[2]

[1]University of the Incarnate Word School of Osteopathic Medicine, San Antonio, TX
[2]University of Incarnate Word Feik School of Pharmacy, San Antonio, TX

Abstract

Disclaimer: The authors have nothing to disclose.

Introduction: Advanced glycation end products (AGEs) are products of nonenzymatic reactions between reducing sugars and proteins, lipids, or nucleic acids and serve as important biomarkers in different research areas like health science, food, and agriculture. Nε-(carboxymethyl)lysine (CML) and pentosidine (PEN) are AGEs and growing evidence suggests association with multiple pathologies such as diabetes, rheumatoid arthritis, osteoarthritis, and arthrofibrosis [1]. Pyridinoline (PYD) and deoxypyridinoline (DPD) are enzymatically produced collagen cross-links found in synovium, ligaments, tendons, bone, and other human tissues [2]. PYD, DPD, PEN, and CML are frequently targeted in studies of musculoskeletal health and no published methods to date have provided a protocol for the identification and quantification of all four analytes together, via high performance liquid chromatography (HPLC). If this could be achieved, this would make this line of investigation more accessible to many more labs across the world. This study reports a method our group developed to identify CML, PEN, PYD, and DPD in a single analysis via ion-pairing HPLC.

Methods: Preparation of standards: CML, PEN were obtained from Cayman Chemical and DPD, PYD from Quidel. Solutions of CML (0.8 mg/mL), PEN (0.8 mg/mL), PYD (0.02 mg/mL), and DPD (0.02 mg/mL) were prepared in 10mM HFBA using a vortex mixer for dissolution and stored at 4°C.

Mobile phase: 10mM HFBA in ultrapure water and HPLC-grade acetonitrile (AcN) were used as mobile phase.

Preparation of CML-FMOC: CML, lacking chromophores for direct detection via UV or fluorescence, was derivatized with 9-fluorenylmethyl chloroformate (FMOC-Cl) for fluorescent detection in the HPLC, following the protocol by Shangguan et al [3]. Briefly, a solution of CML is adsorbed in a bed of alkaline silica gel, and a solution of FMOC-Cl is percolated through for about ten minutes. The bed is rinsed with ethyl acetate to remove the excess of FMOC-Cl and a solution of methanol, water, and acetic acid to remove the analyte FMOC-CML.

HPLC: 40 mL of each standard were injected into a Dionex Ultimate 3000 system programmed for a 16 minute run at 1 mL/min with 5% AcN and 95% 10mM HFBA, changing to 15% and 85%, respectively, for 10 minutes and returning to 5% and 95% for equilibration to a complete run time of 35 minutes. The column used was a Polaris 3 C18 column (Agilent) kept at 45 °C. The column was pre-conditioned through running the 5% AcN and 95% 10mM HFBA solvent system overnight at a flow of 0.3 mL/min.

Results: 40 mL of a mixture of the standards CML-FMOC, DPD, PYD, and PEN was injected, with retention times of 5.72, 8.26, 10.42, and 20.92 minutes, respectively. These retention times were consistent with individual analysis of each standard.

D**iscussion:** The establishment of a unified analytical method for HPLC detection and quantification of CML-FMOC, DPD, PYD, and PEN is an important first step towards our goal of studying the relationship between the presence of these markers in arthrofibrotic synovial tissues following total knee arthroplasty and long-term stability of the implanted replacement. The use of a combined isocratic-gradient protocol eluted the analytes at distinct times and with reasonable analysis lengths. To the best of our knowledge this is the first report of a method capable of analyzing all four compounds in a single HPLC run.

Significance: Historically researchers have used different techniques and assays to identify and analyze the presence and concentration of these target analytes. The identification of these AGEs (CML and PEN) and collagen crosslinks (DPD, PYD), combined, in a single HPLC analysis, offers a relatively affordable alternative for their detection and quantification through the use of a simple HPLC system coupled with a fluorescence detector. This provides more opportunity for pre-clinical and clinical musculoskeletal research into the association of those analytes and disease processes such as arthrofibrosis secondary to joint replacement and diabetes.

References:

1. Verzijl N, DeGroot J, Ben ZC, Brau-Benjamin O, Maroudas A, Bank RA, Mizrahi J, Schalkwijk CG, Thorpe SR, Baynes JW, Bijlsma JW, Lafeber FP, TeKoppele JM. Crosslinking by advanced glycation end products increases the stiffness of the collagen network in human articular cartilage: a possible mechanism through which age is a risk factor for osteoarthritis. Arthritis Rheum. 2002 Jan;46(1):114-23. doi: 10.1002/1529-0131(200201)46:1<114::AID-ART10025>3.0.CO;2-P. PMID: 11822407.
2. Sav NM, Kendirci M, Akin L, Kurtoglu S. Urinary levels of pyridinoline and deoxypyridinoline and bone mineral density in children with type 1 diabetes mellitus. Endocr Res. 2017 Nov;42(4):281-286. doi: 10.1080/07435800.2017.1295982. Epub 2017 Mar 20. PMID: 28318333.
3. Shangguan D, Zhao Y, Han H, Zhao R, Liu G. Derivatization and fluorescence detection of amino acids and peptides with 9-fluorenylmethyl chloroformate on the surface of a solid adsorbent. Anal Chem. 2001 May 1;73(9):2054-7. doi: 10.1021/ac001243c. PMID: 11354490.
4. Takahashi M, Hoshino H, Kushida K, Inoue T. Direct measurement of crosslinks, pyridinoline, deoxypyridinoline, and pentosidine, in the hydrolysate of tissues using high-performance liquid chromatography. Analytical biochemistry. 1995 Dec 10;232(2):158-62. Doi: 10.1006/abio.1995.0002