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High sensitivity HPLC method for determination of the allysine concentration in tissue by use of a naphthol derivative

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ABSTRACT

Common to all fibrotic and metastatic diseases is the uncontrollable remodeling of tissue that leads to the accumulation of fibrous connective tissue components such as collagen and elastin. Build-up of fibrous tissue occurs through the cross-linking of collagen or elastin monomers, which is initiated through the oxidation of lysine residues to form α-aminoadipic-δ-semialdehyde (allysine). To provide a measure of the extent of collagen oxidation in disease models of fibrosis or metastasis, a rapid, sensitive HPLC method was developed to quantify the amount of allysine present in tissue. Allysine was reacted with sodium 2-naphthol-7-sulfonate under conditions typically applied for acid hydrolysis of tissues (6 M HCl, 110 °C, 24 h) to prepare AL-NP, a fluorescent bis-naphthol derivative of allysine. High performance liquid chromatography was applied for analysis of allysine content. Under optimal reaction and detection conditions, successful separation of AL-NP was achieved with excellent analytical performance attained. Good linear relationship (R^2 = 0.994) between peak area and concentration for AL-NP was attained for 0.35–175 pmol of analyte. A detection limit of 0.02 pmol in the standard sample with a 20 μL injection was achieved for AL-NP, with satisfactory recovery from 88 to 100% determined. The method was applied in the quantification of allysine in healthy and fibrotic mouse lung tissue, with the fibrotic tissue showing a 2.5 fold increase in the content of allysine.

1. Introduction

Increased extracellular matrix (ECM) formation and tissue stiffening is a fundamental feature of wound healing, fibrotic diseases, and in the regulation of cancer cell metastasis. Lysyl oxidase (LOX) and its paralogs, LOX-like 1–4 (LOXL1-4) are extracellular, matrix embedded proteins that play a critical role establishing the structural integrity and stability of the ECM through the cross-linking of collagen and elastin fibrils [1,2]. The LOX/LOXL1-4 dependent oxidation of lysine residues on collagen yields α-aminoadipic-δ-semialdehyde (allysine) [3], which undergoes a series of condensation reactions generating the cross-linking structures responsible for collagen and elastin fiber formation [4–9].

During fibrosis, chronic injury leads to the excess accumulation of ECM scar tissue [10,11], and the disruption of normal tissue function, which can lead to organ failure and mortality. Elevated levels of the LOX family is a consistent feature across different organs and tissues types, with increased levels of the enzymes reported in patients with enhanced myocardial stiffness [12], chronic hepatic fibrosis, adriamycin-induced kidney fibrosis[13] and several types of cancer (breast [14,15], head and neck [14], and colorectal [16,17], among others[18]). High levels of LOX expression have similarly been described in mouse models of liver and lung fibrosis [19]. LOX expression has been shown to be an important regulator of tumor progression, with implications for the promotion of cancer cell invasion, metastasis and angiogenesis, as well as in the malignant transformation of solid tumors through remodeling of the tumor microenvironment [20–23]. Secreted LOX has also been shown to be involved in the recruitment of inflammatory cells to distant sites, which contribute to metastasis by initiating the formation of the pre-metastatic niche in breast and liver cancers [24].

The molecular pathways common to ECM remodeling in fibrosis and solid tumor metastasis across different tissue types suggest that the LOX and LOXL1–4 enzymes may be useful targets for developing anti-tumorigenic and anti-fibrotic therapeutics. LOX and LOXL2, in particular, have been the basis of extensive investigation, with LOX identified as a therapeutic target for patients with cancers [14], while LOXL2 has received focus in the monitoring and treatment of fibrotic lung disease

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[25,26]. Over the last few decades several selective inhibitors of LOX, from small molecule inhibitors (e.g. β-aminopropionitrile[27]) to function blocking antibodies, have been developed [28,29], with Simtuzumab, an antibody inhibitor of LOXL2[25] currently under active investigation.

The formation of allysine is a fundamental component in the LOX and LOXL dependent mechanism of ECM deposition during malignancy. Since allysine is present only transiently in tissue during periods of LOX upregulation, we hypothesize that measurement of the allysine content of tissue would provide a way to quantify the fibrogenic activity. This would allow the initial stages of fibrosis and/or metastasis to be identified and provide a quantitative biochemical measure of the effectiveness of LOX and LOXL1-4 targeted therapies.

Quantification of allysine has proven to be challenging as chemical derivatization is required to avoid loss of aldehyde functionality during tissue hydrolysis steps. Early methods included quantification using conventional amino acid chromatography analyzers following either protein reduction using [^{3}H]$NaBH_4$ to give hydroxynorleucine/chlornorleucine, oxidation with performic acid to give α-aminoadipic acid or reaction with [^{14}C]NaCN and NH_3 to give α,ε-diaminopimelic acid and ε-hydroxy-α-aminopimelic acid [30,31]. More recently in the context of quantifying protein oxidation, several methods have been described in biological samples with GC–MS, HPLC or LC–MS used for quantification [32–34]. Reductive amination with $NaCNBH_3$ in the presence of *p*-aminobenzoic acid has been performed to produce an acid stable fluorescent allysine derivative that can be detected by HPLC following hydrolysis by either conventional thermal heating or microwave irradiation [35,36]. Reductive amination with fluoresceinamine has also been reported but the fluorescein-allysine conjugate was reported to be unstable during acid hydrolysis of the protein [37]. Requena et al. have developed methods based on GC–MS analysis with isotopic dilution for determination of allysine following reduction with $NaBH_4$ [38]. The equilibrium between allysine and piprideine-6-carboxylate (P6C) has provided an indirect means to estimate allysine concentrations using LC–MS/MS quantification of P6C [39,40]. While these methods provide accurate assessments of the concentrations of allysine present in soluble proteins samples they have not been extended to analyzing whole tissue samples. Methods to quantify the allysine content present in the insoluble collagen matrix include labeling whole tissue samples with phenol and *p*-cresol [41–43]. Umeda et al. show that under conditions suitable for tissue digestion (110 °C, 6 M HCl, 24 h), allysine is derivatized with 2 equivalents of *p*-cresol to yield a bis–*p*-cresol condensation derivative, termed 'APC', enabling quantification of allysine by HPLC-UV analysis. A limitation of the Umeda's protocol is its low sensitivity associated with the detection mode.

In this paper we describe a new procedure that modifies the protocol of Umeda et al. to use 2-naphthol-7-sulfonate for derivatization of allysine with a fluorescent bis-naphthol. The new allysine derivative, 'AL-NP', is stable under the conditions of hydrolysis and allows quantification of allysine in whole tissue with an eight-fold improved sensitivity compared to the *p*-cresol method. Indeed, we showed that when using 2-naphthol-7-sulfonate it is possible to quantify allysine by HPLC with detection sensitivity as low as pmoles using milligram quantities of whole tissue digest without the prior need to isolate the ECM components.

2. Materials and methods

2.1. Reagents and materials

All reactants and reagents were of the highest purity. HPLC-grade acetonitrile was obtained from EMD Millipore (Billerica, MA, USA). Water was purified with a Milli-Q system (Millipore, Billerica, MA, USA). L-Allysine ethylene acetal (> 98%), potassium hydrogen phthalate (> 99%) and fluorescein (99%) were supplied by Sigma (Sigma-Aldrich, Saint Louis, MO, USA). Trifluoroacetic acid (99.5%) was purchased from Alfa Aesar (MA, USA), formic acid (99.0%) from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ, USA) and sodium 2-naphthol-7-sulfonate (> 98%) from TCI America (PA, USA). Analytical grade methanol and chloroform were obtained from VWR (PA, USA).

2.2. NMR

NMR spectra were recorded on a Varian 500 NMR system equipped with a 5 mm broadband probe (^{1}H NMR: 499.81 MHz, ^{13}C: 125.68 MHz). ^{1}H resonance assignments were obtained using ^{1}H–^{1}H correlated spectroscopy.

2.3. HPLC

Mobile phases were prepared by adding 1 mL of trifluoroacetic acid to 999 mL water for Solvent A, 1 mL of trifluoroacetic acid to 999 mL HPLC grade acetonitrile for Solvent B, 1 mL of formic acid to 999 mL water for Solvent C and 1 mL of formic acid to 999 mL HPLC grade acetonitrile for Solvent D. The mobile phases were filtered under a vacuum through a 0.45 mm nylon filters and degassed continuously with an on-line degasser.

2.3.1. Preparative HPLC

Purification of AL-NP was performed using a MetaChem Technologies Inc. LUNA $C^{18}(2)$ column (250 × 21.2 mm, particle size: 10 μm). UV detection was at 220, and 254 nm. The flow rate was 15 mL/min and a gradient method was employed using Solvents A and B: 0–2 min hold at 5% B, 2–24 min gradient to 95% B, 24–25 min hold at 95% B, 25–28 min gradient to 5% B, 28–30 min re-equilibrate at 5% B.

2.3.2. HPLC–MS

HPLC–MS analysis for purity and identity was carried out using an Agilent 1260 LC system coupled to an Agilent Technologies 6130 Quadrupole MS system. The LC method used a Phenomenex Luna $C^{18}(2)$ column (100 × 2 mm, particle size: 5 μm). The flow rate was 0.8 mL/min, with UV detection at 220, 254 and 280 nm. A gradient chromatographic method was employed using solvents C and D: 0–1 min hold at 5% D, 1–9 min gradient to 95% D, 9–10 min hold at 95% D, 10–12 min gradient to 5% D, 12–15 min re-equilibrate at 5% D.

2.3.3. Analytical HPLC-FL

HPLC analysis was carried out on an Agilent 1260 system with an Agilent 1260 FLD fluorescence detector (λ_{ex} = 254 nm and λ_{em} = 310 nm for AL-NP; λ_{ex} = 490 nm and λ_{em} = 510 nm for fluorescein used as internal standard). Control of the HPLC system and data collection was by Agilent OpenLAB CDS ChemStation software (version 1.90). A C^8 analytical Discovery® column (25 mm × 4 mm, 5 μm particle size) was used. The flow rate was 1 mL/min and the oven temperature was set to 25 °C. The injection volume was set at 20 μL. A gradient chromatographic method was employed using solvents A and B: 0–40 min; 5–30% solvent B, 40–42 min; 30–95% solvent B, 42–45 min; 95% solvent B, 45–47 min; 95-5% solvent B, 47–50 min; 5% solvent B.

2.4. Synthesis of allysine-bis-p-naphthol derivative

L-Allysine ethylene acetal (25 mg, 0.13 mmol) in 6N HCl (10 mL) containing 2-naphthol-7-sulfonate (325 mg, 1.32 mmol) was heated at 110 °C for 24 h. The reaction was cooled to r.t., neutralized with 6 N NaOH, filtered and purified by preparative HPLC to yield a pale brown solid (65.5 mg, 87%).

^{1}H NMR (D_2O, 500 MHz, 25 °C): 8.25 (s, 2H, ArH), 7.86 (d, 2H, *J* = 7.2 Hz, ArH), 7.73 (d, 2H, *J* = 7.2 Hz, ArH), 7.43 (m, 2H, ArH), 6.67 (m, 2H, ArH), 4.41 (s, 1H, C*H*(NH_2)CO_2H), 3.14 (t, 1H, *J* = 7.4 Hz, C*H*), 1.49 (m, 2H, CH_2), 1.16 (pseudo dt, 1H, CH*H*'), 1.06