Microchemical Journal 137 (2018) 85–89

Contents lists available at ScienceDirect

Microchemical Journal

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

Development and validation of high performance liquid chromatographic method for determination of gentisic acid and related Renal Cell Carcinoma biomarkers in urine

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ARTICLE INFO

Article history:
Received 18 September 2017
Accepted 25 September 2017
Available online 28 September 2017

Keywords:
Renal Cell Carcinoma
Biomarkers
Metabolomics
4-Hydroxybenzoate
Reversed phase liquid chromatography
Water rich mobile phases

ABSTRACT

A reversed phase liquid chromatographic (RPLC) method was developed to simultaneously detect and quantify creatinine, quinolinic acid, gentisic acid and 4-hydroxybenzoic acid in urine. These four bio-markers are present in relatively high concentrations in urine. Using a 5% methanol in water mobile phase with 0.6% acetic acid and a Zorbax C_{18} column, baseline resolution for all four biomarkers in synthetic urine was achieved. Better resolution was obtained for the separation of these four compounds when water rich mobile phases were used. Detection of the four biomarkers in urine using the proposed RPLC method is limited by background from the urine matrix for the later eluting compounds and from the dead marker for earlier eluting compounds.

1. Introduction

Renal Cell Carcinoma (RCC) is a disease that causes malignant cells to form in tubules of the kidney [1]. RCC, the third most common form of genitourinary cancer, is a disease which is asymptotic in its initial stages. Patients are often diagnosed with RCC at the terminal or metastatic stage. At present, RCC has the highest mortality rate of any urological tumor [2]. The most common symptoms of RCC are a lump on the belly or back, blood in the urine; lower back pain, weight loss due to loss of appetite, fever, lethargy, anemia, night sweats; and high calcium levels in blood [3]. When these symptoms appear, the survival rate for the patient is low. To improve the prognosis for patients suffering from RCC, early detection and treatment through the development of a methodology based on analysis of biological fluids (e.g. urine and plasma) for molecular markers characteristic of RCC in its early stages is desirable. An obvious advantage of this approach to cancer prescreening is that urine and plasma samples are inexpensive and readily accessible.

Currently, RCC diagnosis is based on magnetic resonance imaging (MRI), computerized tomography (CT) scans, contrast-enhanced ultrasonography (CEUS), and position emission tomography-computed tomography (PET- CT) [4]. These imaging techniques are both accurate and effective. However, these methods require continued surveillance of small indolent renal masses, which is costly with attendant side effects. Gonzalez and coworkers [5] have reported in the United States that approximately 29,000 cases of malignancy will occur each year from exposure to radiation from radiologic imaging. Furthermore, routine screening of the general population using these methods is not cost-effective. Therefore there is high demand for non-invasive RCC diagnostic techniques which can be used for routine screening.

Urine has considerable value as a diagnostic biological fluid because it contains large amounts of metabolites. Also, the non-invasive nature in which urine is collected makes it an ideal biological fluid for cancer prescreening [6]. As cancer develops in a patient, there is an additional energy demand on the patient's body, and cancer cells activate specific metabolic pathways to compensate for this energy demand. Therefore the patient's metabolic profile may contain reliable biomarker for early cancer detection.

It has been previously reported by Kim and coworkers [7] that quinolinic acid (pyridine-2, 3-dicarboxylic acid), 4-hydroxybenzoic acid and gentisic acid (2, 5-dihydroxybenzoic acid) levels in the urine of RCC patients is differently expressed when compared to urine from normal controls. In this study, Kim and coworkers analyzed urine samples from 29 RCC patients and from 33 patients with other urological conditions. Ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS) and gas chromatography/mass spectrometry (GC/MS) was used to conduct this untargeted metabolomic profiling and 274 known metabolites were identified in this study. Among the metabolites identified, quinolinic acid, gentisic acid and 4-hydroxybenzoic acid were found to exhibit the largest

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https://doi.org/10.1016/j.microc.2017.09.024
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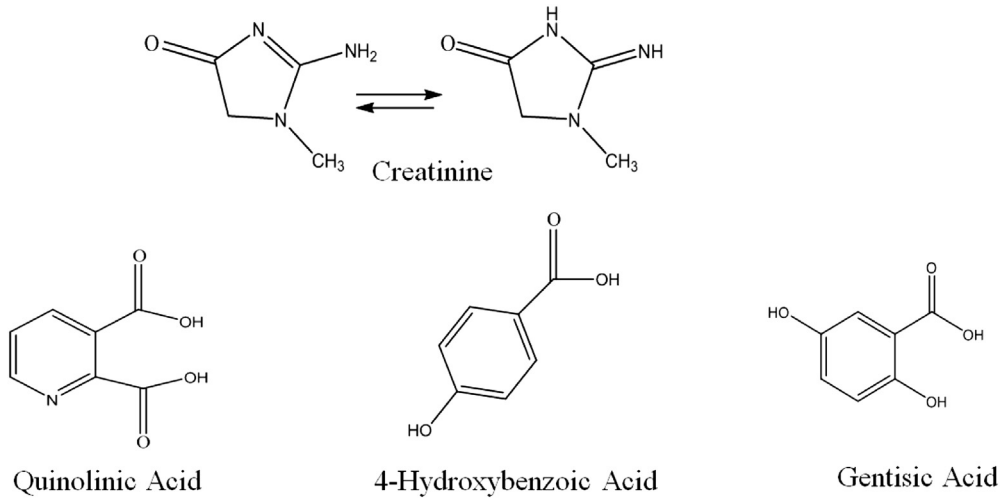

Fig. 1. The chemical structures of the four biomarkers investigated in this study.

degree of expression compared to the controls (at $p < 0.05$). Quinolinic acid is an intermediate in several metabolic pathways including one governing Tryptophan metabolism. Quinolinic acid is also involved in the production of nicotinate D-ribonucleotide, which is a precursor to NAD^+ [7,8]. Gentisic acid and 4-hydroxybenzoic acid play an important role in benzoate degradation and may also prevent lipoprotein oxidation in cancer cells.

Having identified the targeted metabolites, an untargeted metabolomic analysis would not be convenient nor sufficiently accurate to quantify these bio-markers in a clinical study. Clearly, there is a need for an accurate, fast, and inexpensive analytical method to quantify quinolinic acid, gentisic acid and 4-hydroxybenzoic acid in urine. The only reported procedure in the literature to quantify these three biomarkers simultaneously was developed by Ma [9] which utilizes liquid chromatography/tandem mass spectrometry (LC/MS/MS). However, the cost of analysis per sample using LC/MS/MS is high due to the expense of the instrument and its maintenance cost. For this reason, LC/MS/MS is typically not available in most clinical laboratories.

In this study, a reversed phase liquid chromatographic (RPLC) procedure was developed to simultaneously detect and quantify quinolinic acid, gentisic acid and 4-hydroxybenzoic acid in urine. These three bio-markers are present in relatively high concentration in urine. In the LC/MS/MS method previously reported by Ma, each urine sample was diluted one-hundred fold prior to analysis [9]. Furthermore, Ma normalized the chromatograms using specific gravity to take into account renal dilution. In the current study, we accounted for the renal dilution of the urine by including creatinine as a targeted analyte. The concentration of creatinine corresponds closely to urine dilution, so its levels should be closely monitored in any urinary biomarker analysis. A biomarker-to-creatinine ratio will give more meaningful results than the concentration of the biomarker alone [10,11].

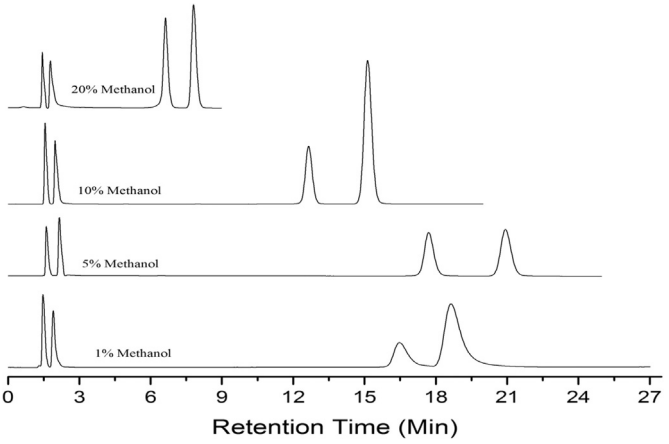

Fig. 2. Chromatograms of the biomarker test mixture (for creatinine, quinolinic acid, gentisic acid and 4-hydroxybenzoic acid) for 20% methanol, 10% methanol, 5% methanol and 1% methanol in water with 0.6% acetic acid. The concentration of each compound in the test mixture was 200 ppm.

2. Experimental

The four compounds investigated in this study, creatinine, quinolinic acid, gentisic acid and 4-hydroxybenzoic acid were purchased from Sigma Aldrich and were used as received. The chemical structures of these four compounds are shown in Fig. 1. Stock solutions of each compound were prepared by weighing and dissolving the corresponding amount in doubly distilled water with 0.6% acetic acid. Standard mixtures of these four compounds were prepared by dilution to the appropriate concentration also using 0.6% acetic acid in doubly distilled water. As these compounds were weakly retained by the C_{18} alkyl bonded phase, it was necessary to use water as the solvent to prepare the test mixtures. If a stronger solvent such as methanol was used, the test mixture would not have been deposited onto the head of the column as a thin plug during sample injection with the result being increased band broadening. For the preparation of the stock solutions and the test mixtures, glacial acetic acid which was used to acidify these solutions was obtained from Pharmco. Doubly distilled water was prepared using a Barnstead Nano-Pure II System (Barnstead International, Dubuque, IA). For preparation of the urine samples, artificial urine (Flinn Scientific) was diluted five times with doubly distilled water containing 0.6% acetic acid and then spiked with the appropriate amount of the test mixture to yield 20 ppm, 100 ppm and 700 ppm mixtures.

The chromatographic studies were performed using a Varian High Performance Liquid Chromatograph equipped with a Zorbax 5 μm Eclipse-XDB C_{18} 80 A^0 column (150 × 4.6 mm), Shimadzu column oven, ProStar reciprocating pump and a diode array detector. The dead time, which was determined by injecting different solutions (methanol, water, or KNO_3) onto the Zorbax column, was calculated to be 1.41 ml. All k′ values reported in this study were averages of triplicate determinations and deviations for individual k′ values were not >1%.

Liquid chromatography/mass spectrometry (LC-MS) analysis was performed using a Shimadzu 2010EV LC-MS equipped with electrospray ionization. LCMS solution software (Shimadzu) was used for instrument control and for data acquisition and analysis. Liquid chromatographic separations on the LC-MS were carried out with a Shimadzu Premier C_{18} 3 μm column (100 × 4.6 mm).