Published in final edited form as:

Microchem J. 2018 March ; 137: 85–89. doi:10.1016/j.microc.2017.09.024.

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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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 C₁₈ 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.

Keywords

Renal Cell Carcinoma; Biomarkers; Metabolomics; 4-Hydroxybenzoate; Reversed phase liquid chromatography; water rich mobile phases

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 asymptomatic 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

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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 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 bio-markers 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].

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 Figure 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 C₁₈ 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 5µm Eclipse-XDB C₁₈ 80A⁰ column (150 x 4.6mm), Shimadzu column oven, ProStar reciprocating pump and a diode array detector. The dead time, which was determined by injecting different solutions (methanol, water, or KNO₃) 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 greater than 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 C₁₈ 3 μ m column (100 x 4.6mm).

All HPLC mobile phases were prepared using reagent grade glacial acetic acid (Pharmco) and two HPLC grade solvents: methanol (Thermo-Fisher) and water (Thermo-Fisher). Water rich mobile phases containing 5%, 2.5%, and 1% methanol were prepared with a transfer pipette because of the small volume of methanol needed to prepare these solutions. All mobile phase solutions were degassed prior to their use. All mobile phase solutions were percolated through the Zorbax column at a flow rate of 1.0 ml/minute for several hours to ensure reproducible column wetting. This flow rate was used in all of the studies because of the desire to develop an inexpensive RPLC method. For the same reason, the temperature of the column was ambient. The injection volume for each compound and test mixture was 5 μ l.

Results and Discussion

A series of chromatograms were run to illustrate the advantages of using methanol-water mobile phases with 0.6% acetic acid for the separation of creatinine, quinolinic acid, gentisic acid and 4-hydroxybenzoic acid. Figure 2 shows chromatograms of the test mixture for 20% methanol, 10% methanol, 5% methanol and 1% methanol. The concentration of each compound in the test mixture was 200ppm. For the 20% methanol, 10% methanol, and 5% methanol runs, each compound of the test mixture exhibited classical RPLC hydrophobic behavior: retention time increased as the organic modifier concentration in the mobile phase decreased. For the 1% methanol run, the decrease in retention time of the test mixture when compared to the 5% methanol run can be attributed to extrusion of the mobile phase and sample molecules from the particle pores where most of the surface area of the C₁₈ alkyl bonded phase is located [12]. The net result was a loss in retention due to a decrease in accessible contact surface area.

$$\ln k' = \ln k_w - B\phi \quad (1)$$

In RPLC, insight into the factors that may influence retention can be gained by a thorough analysis of retention data for a set of structurally related compounds using Equation 1, where ϕ is the volume percentage of organic modifier in the mobile phase, B is a measure of the interaction of the solute with the mobile phase and $\ln k_w$ is the logarithm of the retention factor for the compound in a purely aqueous media. Both B and $\ln k_w$ are constants characteristic of the solute.

Figure 3 shows a plot of $\ln k'$ versus ϕ for quinolinic acid, gentisic acid and 4-hydroxybenzoic acid. Creatinine was excluded from this analysis because it co-eluted with the dead marker. Eight methanol-water mobile phases with 0.6% acetic acid were used to generate these plots: 30%, 25%, 20%, 15%, 10%, 5%, 2.5%, and 1% methanol in water. For all three compounds, the 1% run was excluded from the regression analysis and the 1% and 2.5% runs were excluded from the regression analysis for 4-hydroxybenzoic acid and gentisic acid as this data did not follow the trend expected for organic compounds from classical RPLC

retention behavior. Exclusion of only the 1% run from the regression analysis for quinolinic acid can probably be attributed to this compound being less hydrophobic than 4-hydroxybenzoic acid and gentisic acid, which in turn would make it more accessible to the pores when using water rich mobile phase. Both B and $\ln k_w$ in Equation 1 for these three compounds can be correlated to their water solubility and their log P values (see Table 1).

The 5% methanol in water mobile phase with 0.6% acetic acid was selected for the RPLC analyses of the four renal cell carcinoma biomarkers investigated in this study. Table 2 lists the number of plates generated by this mobile phase for each compound of the test mixture. The lower number of plates obtained for creatinine and quinolinic acid compared to gentisic acid and 4-hydroxybenzoic acid can be attributed to extra column band broadening by the detector. The 5% methanol in water mobile phase was selected for the RPLC analyses reported in this study because of the better separation achieved for creatinine and quinolinic acid and lower interference from the urine matrix for quinolinic acid. Figure 4 shows two chromatograms obtained using the 5% methanol in water mobile phase: the test mixture and the artificial urine. LC-MS analysis of the artificial urine, which was run in negative ion mode, indicated the presence of creatinine and 4-hydroxybenzoic acid based on the m/z value of the molecular ion for the two peaks identified in the chromatogram of artificial urine (see Figure 4).

Calibration curves for creatinine, quinolinic acid, gentisic acid and 4-hydroxybenzoic acid shown in Figures 5 through 8 were developed from standards prepared using doubly distilled water with 0.6% acetic acid. For creatinine, quinolinic acid, and gentisic acid, 232nm was selected for the development of the calibration plots as this wavelength corresponded to an absorbance maximum, whereas 252nm was selected for 4-hydroxybenzoic acid due to its high ϵb value. Calibration curves for both creatinine and quinolinic acid did not pass through the origin which can be attributed to the uncompensated background from the dead marker as these compounds eluted with the dead marker or near the dead marker, whereas the calibration curves for both gentisic acid and 4-hydroxybenzoic acid, which are quite distant from the dead marker pass through the origin.

Three urine samples containing 20ppm, 100ppm, and 700ppm of each component of the test mixture were prepared using synthetic urine to mimic detection of the four renal cancer biomarkers by RPLC. Each urine sample was run in triplicate, and the four calibration curves (see Figures 5 through 8) developed from standards prepared using doubly distilled water with 0.6% acetic acid were used to quantify the amount of each biomarker in the urine samples. Table 3 lists the recovery and relative standard deviation obtained for each urine sample. The relative standard deviations for these analyses generally decreased with increasing biomarker concentration. For all four biomarkers, the recovery was near 100% for the most concentrated urine sample. Both creatinine and 4-hydroxybenzoic acid were plagued by interference from the urine matrix, which would explain their high reported recovery rates for the 20ppm urine sample. Quinolinic acid and gentisic acid, by comparison, showed the opposite trend – an increase in recovery rate with increasing concentration – this is a result of the urine matrix not interfering with the detection of these two compounds. The high relative standard deviation of quinolinic acid at 20ppm indicates that the detection limit of this compound is being approached using the proposed RPLC

method. For creatinine, quinolinic acid, and 4-hydroxybenzoic acid, it is evident that background from the urine matrix or the dead marker will limit the detection of these compounds in urine.

Acknowledgments

This study was supported by 5P20GM103447-18 awarded by the National Institutes of Health. The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the National Institutes of Health.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics 2015. CA: A Cancer Journal for Clinicians. 2015; 65(1):5–29. [PubMed: 25559415]
2. European Network of Cancer Registries. European Incidence Database V2.3, 730 entity dictionary. 2001. Eurocim Version 4.0.
3. Abbasi A, Johnson TV, Master VA. Minor Symptoms are Suggestive of Metastatic Renal Cell Carcinoma if C - Reactive Protein Remains High After Curative Nephrectomy. J Clin Oncol. 2011; 29(6):e133–134. [PubMed: 21115862]
4. Agabegi, ED., Agabegi, SS. Step-Up to Medicine, Chapter 7 (Discussion of the Renal and Genitourinary System). Lippincott Williams & Wilkins; Hagerstown, MD: 2008.
5. Gonzalez BA, Mahesh M, Kim KP, Bhargavan M, Lewis R, Mettler F, Land C. Projected Cancer Risks From Computed Tomographic Scans Performed in the United States in 2007. Arch Intern Med. 2009; 169(22):2071–2077. [PubMed: 20008689]
6. Catchpole G, Platzer A, Weikert C, Kempkensteffen C, Johannsen M, Krause H, Jung K, Miller K, Willmitzer L, Selbig J, Weikert S. Metabolic Profiling Reveals Key Metabolic Features of Renal Cell Carcinoma. J Cell Mol Med. 2011; 15(1):109–118. [PubMed: 19845817]
7. Kim K, Taylor SL, Ganti S, Guo L, Osier MV, Weiss RH. Urine Metabolomic Analysis Identifies Potential Biomarkers and Pathogenic Pathways in Kidney Cancer. Omics. 2011; 15(5):293–303. [PubMed: 21348635]
8. Schwartz R, Whetsell WO Jr, Mangano RM. Quinolinic acid: An Endogenous Metabolite that Produces Axon-Sparing Lesions in Rat Brain. Science. 1983; 219(4582):316–318. [PubMed: 6849138]
9. Chen S, Burton C, Kaczmarek A, Shi H, Ma Y. Simultaneous Determination of Urinary Quinolate, Gentisate, 4-Hydroxybenzoate, and α -Ketoglutarate by High-Performance Liquid Chromatography-Tandem Mass Spectrometry. Anal Methods. 2015; 7(16):6572–6578.
10. Gamagedara S, Gibbons S, Ma Y. Investigation of Urinary Pteridine Levels as Potential Biomarkers for Noninvasive Diagnosis of Cancer. Clin Chim Acta. 2011; 412(1–2):120–128. [PubMed: 20869359]
11. Gamagedara S, Shi H, Ma Y. Quantitative Determination of Taurine and Related Biomarkers in Urine by Liquid Chromatography-Tandem Mass Spectrometry. Anal Bioanal Chem. 2012; 402(2): 763–770. [PubMed: 22038588]
12. Walter TH, Iraneta P, Capparella M. Mechanism of Retention Loss when C₈ and C₁₈ HPLC Columns are used with Highly Aqueous Mobile Phases. J Chromatog A. 2005; 1075:177–183. [PubMed: 15974131]
13. Foley JP, Dorsey JG. Equations for Calculation of Chromatographic Figures of Merit for Ideal and Skewed Peaks. Anal Chem. 1983; 55:730–737.

Highlights

- This study demonstrates the advantages of using water-rich mobile phases in RPLC for biomarker analysis.
- An inexpensive RPLC method has been developed to detect Renal Cell Carcinoma in its earlier stages.
- Preliminary studies using synthetic urine are encouraging.

Figure 1.

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

Figure 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 200ppm.

Figure 3. Plots of $\ln k'$ versus ϕ for a) quinolinic acid, b) gentisic acid and c) 4-hydroxybenzoic acid.

Figure 4. Two chromatograms obtained with the 5% methanol in water mobile phase: the test mixture and the artificial urine.

Figure 5.

Calibration curve for creatinine. The concentration of the standards used to develop this calibration curve using the RPC method were 5 ppm, 10 ppm, 50 ppm, 70 ppm, 100 ppm, 250 ppm, 500 ppm, 700 ppm and 1000 ppm. Each standard was analyzed in triplicate.

Figure 6.

Calibration curve for quinolinic acid. The concentration of the standards used to develop this calibration curve using the RPC method were 5 ppm, 10 ppm, 50 ppm, 70 ppm, 100 ppm, 250 ppm, 500 ppm, 700 ppm and 1000 ppm. Each standard was analyzed in triplicate.

Figure 7.

Calibration curve for gentisic acid. The concentration of the standards used to develop this calibration curve using the RPC method were 5 ppm, 10 ppm, 50 ppm, 70 ppm, 100 ppm, 250 ppm, 500 ppm, 700 ppm and 1000 ppm. Each standard was analyzed in triplicate.

Figure 8. Calibration curve for 4-hydroxybenzoic acid. The concentration of the standards used to develop this calibration curve using the RPC method were 5 ppm, 10 ppm, 50 ppm, 70 ppm, 100 ppm, 250 ppm, 500 ppm, 700 ppm and 1000 ppm. Each standard was analyzed in triplicate.

Table 1Parameters from Regression Analysis for $\ln k'$ versus ϕ

Compounds	Water Solubility	Log P	$\ln k_w$	B
Quinolinic Acid	11mg/ml	0.15	-0.68871 ± 0.03794	-0.04501 ± 0.0021
Gentisic Acid	5mg/ml	1.42	2.67733 ± 0.04344	-0.06823 ± 0.00223
4-hydroxybenzoic acid	5mg/ml	1.67	2.87847 ± 0.04159	-0.06793 ± 0.00214

Table 2Number of Plates^{1, 2} Generated by 5% Methanol Mobile Phase

Compounds	Retention Time	Number of Theoretical Plates	Asymmetry Values
Creatinine	1.61 minutes	1242	2.48
Quinolinic Acid	2.15 minutes	1627	2.93
Gentisic Acid	17.64 minutes	10092	1.20
4-hydroxybenzoic acid	20.86 minutes	10634	1.20

¹ Plate calculations performed using the Foley-Dorsey method [13].² Each plate calculation is an average of triplicate determinations.

Table 3

Recovery and Relative Standard Deviation for each Urine Sample

Compounds	Low concentration (20 ppm)		Middle concentration (100 ppm)		High concentration (700 ppm)	
	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
Creatinine	7.93	410	2.35	163	0.37	108
Quinolinic Acid	15.57	82.5	3.76	89	0.3	97
Gentisic Acid	4.37	90.7	4.27	92	8.08	97
4-Hydroxybenzoic Acid	7.65	166	1.01	113.4	1.04	108