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A novel method of liquid chromatography–tandem mass spectrometry combined with chemical derivatization for the determination of ribonucleosides in urine

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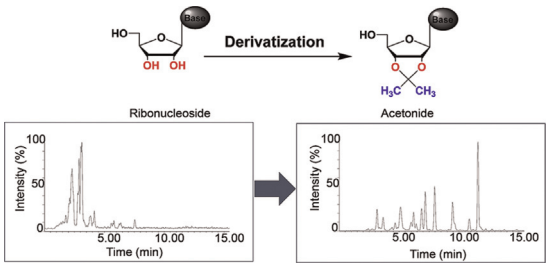
HIGHLIGHTS

- A simple, robust and low-cost derivatization method was reported for ribonucleoside determination for the first time.
- Improvement of separation and enhancement of sensitivity were achieved by using the derivatization approach.
- Isotope labeling method with acetone-d_6 and multivariate statistical analysis facilitated ribonucleoside identification.
- Application of the method enabled the positive identification of 56 ribonucleosides.

GRAPHICAL ABSTRACT



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ABSTRACT

Ribonucleosides are the end products of RNA metabolism. These metabolites, especially the modified ribonucleosides, have been extensively evaluated as cancer-related biomarkers. However, the determination of urinary ribonucleosides is still a challenge due to their low abundance, high polarity and serious matrix interferences in urine samples. In this study, a derivatization method based on a chemical reaction between ribonucleosides and acetone to form acetonides was developed for the determination of urinary ribonucleosides. The derivative products, acetonides, were detected by using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The methodological evaluation was performed by quantifying four nucleosides for linear range, average recovery, precision, accuracy and stability. The validated procedures were applied to screen modified ribonucleosides in urine samples. Improvement of separation and enhancement of sensitivity were obtained in the analysis. To identify ribonucleosides, inexpensive isotope labeling acetone (acetone-d_6) and label-free acetone were applied to form ordinary and deuterated acetonides, respectively. The two groups of samples were separated with orthogonal partial least squares (OPLS). The ordinary and deuterated pairs of acetonides were

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symmetrically distributed in the S-plot for easy and visual signal identification. After structural confirmation, a total of 56 ribonucleosides were detected, 52 of which were modified ribonucleosides. The application of derivatization, deuterium-labeling and multivariate statistical analysis offers a new option for selective detection of ribonucleosides in biological samples.

1. Introduction

Ribonucleic acid (RNA) molecules play an important regulatory role in cell biology. RNA copies the genetic information from deoxyribonucleic acid (DNA), and then translates them into proteins. After completing its assigned tasks, RNA is catabolized to nucleoside level. The main composition of these end products or unmodified ribonucleosides, namely adenosine (A), guanosine (G), urine (U) and cytidine (C), were recycled to synthesize new RNA. However, the modified ribonucleosides that cannot be catalyzed by the phosphorylases are excreted from the cell into the urine.

The levels of urinary ribonucleosides are fairly constant in normal healthy individuals with little difference [1]. The excretion of ribonucleosides is not affected by age and diet [2]. But elevated levels of ribonucleosides were observed in patients with physical illness, such as urogenital cancer [3,4], hepatocellular carcinoma [5,6], breast cancer [7], acquired immunodeficiency diseases (AIDS) [8,9] and severe combined immunodeficiency diseases (SCID) [10]. Therefore, the levels of urinary ribonucleosides can be considered as a signal of disease status, especially as potential biomarkers for cancer diagnosis.

Many methods have been developed for the analysis of urinary ribonucleosides. The early used methods included high performance liquid chromatography (HPLC) coupled with UV detector [11] and enzyme-linked immunosorbent assay (ELISA) [12]. The currently favored methods are chromatography or capillary electrophoresis coupled with mass spectrometry (MS) [13–15]. Although MS is more specific and sensitive than UV detector, the extraction and enrichment methods are still necessary for the analysis of ribonucleosides due to the complex components of biological samples and low abundance of ribonucleosides. For example, on-line extraction was performed by connecting a pre-column and an analytical column via a six-way switching valve [16]. For off-line extraction, solid phase extraction (SPE) is the most common pretreatment approach for ribonucleosides [17,18]. The SPE methods can pre-concentrate ribonucleosides but their specificity is poor. Affinity adsorbent approaches based on boronate materials can selectively capture compounds with *cis–diol* groups for the pretreatment of ribonucleosides. SPE packed column [19,20] and magnetic nanoparticles [21] binding boronate groups have been used for the selective enrichment of modified ribonucleosides. These methods have good specificity for extraction of group of ribonucleosides. However, the subsequent separation and determination of intact ribonucleosides, especially the isomers, on reversed-phase LC were often challenging due to their extremely high polarity. Therefore, it is necessary to develop new methods for the determination of these metabolites.

Compounds containing *cis–diol* groups can react with acetone to form acetonides (isopropylidene ketal). The volatility of acetonides is much higher than the substrates. Therefore, this reaction has been used as a derivatization method for the analysis of carbohydrates by using GC [22,23]. Nucleosides containing *cis–diol* groups can also react with acetone to form isopropylidene-ribonucleosides, which has been applied to protect group of *cis–diol* in organic synthesis [24]. However, the application of this specific reaction for the determination of ribonucleosides has not yet been reported. In addition, in the analysis of metabolites, it is a challenge to determine the structure of unknown compounds, especially isomers. Using isotope to selectively label target metabolites can effectively improve the accuracy of structure identification. In this work, a derivatization method based on the reaction of ribonucleosides with acetone was established for urinary ribonucleosides analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The specificity of detection was achieved from the use of acetone-d_6 to label the target compounds. Multivariate statistical analysis method was applied for aided identification of ribonucleosides.

2. Material and methods

2.1. Chemicals

Ribonucleosides were purchased from Sigma–Aldrich (St. Louis, MO, USA), including guanosine (≥98%, G), adenosine (≥99%, A), cytidine (≥99%, C) and uridine (≥99%, U). 8-Chloroguanosine (8ClG, internal standard, IS) was purchased from Carbosynth (Berkshire, UK). Hexadeuteroacetone (99.9 atom% D, acetone-d_6), formic acid (HPLC grade) and 70% perchloric acid ($HClO_4$) were also purchased from Sigma–Aldrich. HPLC grade methanol and acetone were obtained from Merck (Dermstadt, Germany). Acetone was used directly without further drying process. Water was purified by using a Milli-Q system (Millipore, Milford, MA).

2.2. Optimization of derivatization conditions

A nucleoside, guanosine (G) was used as an example to optimize the derivatization reaction conditions. General procedure: to a solution of G (5.00 mg) in 1.0 mL of acetone was treated with $HClO_4$ (10 μL, 70% in water) at −20 °C for 30 min. The resulting solution was neutralized with K_2CO_3. The suspension was centrifuged at 12,000 × *g* for 10 min at 4 °C to remove the precipitation. The supernatant was dried with a stream of nitrogen. The residue was reconstituted with 1.0 mL methanol/water (1:1, v/v). The solution was diluted to 5 μg mL^{-1} and analyzed to determine the reaction efficiency, which was defined as a percent of peak area of reaction products in total peak area. An ACQUITY-Ultra Performance Liquid Chromatography system (UPLC, Waters, USA) equipped with a PDA detector was used for chromatographic measurements. The UPLC–PDA conditions are described in Supplementary material.

The reaction was performed under different temperature, including 20, 4, 0 and −20 °C and different concentration of 70% $HClO_4$ (0.01%, 0.25%, 0.50%, 1.00%, 2.50% and 5.00%, respectively; percentage of acetone in volume). The volume of acetone was determined by quantifying A, G, U and C in urine samples after they were reacted with acetone. Dry residues of 100 μL urine samples were reacted with 300, 400, 500, 600, 700 and 800 μL acetone, respectively. The levels of the four nucleosides were determined by following the method described below which was used for method validation.

2.3. Sample preparation

To perform derivatization, 100 μL urine of sample was dried with an Ilshin Lab freeze dryer (Ilshin Co., Ltd., USA) for 5 h. 600 μL of acetone was added to the residue combined with 6 μL $HClO_4$. The mixture was vigorous vortex for 30 s and set aside at −20 °C for