Chapter 1

Detection of RNA Modifications by HPLC Analysis and Competitive ELISA

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Abstract

Over 100 different RNA modifications exist that are introduced posttranscriptionally by enzymes at specific nucleotide positions. Ribosomal RNA (rRNA) and transfer RNA (tRNA) exhibit the most and diverse modifications that presumably optimize their structure and function. In contrast, oxidative damage can lead to random modifications in rRNA and messenger RNA (mRNA) that strongly impair functionality. RNA modifications have also been implicated in avoiding self-RNA recognition by the immune system or immune evasion by pathogens. Here, we describe the detection of RNA modifications by HPLC analysis and competitive ELISA.

Key words HPLC, Competitive ELISA, RNA, Nucleoside modification, Base methylation, 2′-O-Methylation, N1-Methylguanosine, 2′-O-Methylguanosine, 8-Hydroxyguanosine (8-OHG)

1 Introduction

Over 100 different RNA modifications have been identified and exist in all three kingdoms of life. A comprehensive listing of post-transcriptionally modified RNA nucleosides is found in the RNA Modification Database (http://mods.rna.albany.edu/) [1].

Especially ribosomal and transfer RNA (rRNA and tRNA) are abundantly modified. Examples are 2′-O-ribose methylation, base methylation, and the occurrence of pseudouridine. In the case of 2′-O-ribose methylation of rRNA, the methyltransferase (2′-O-MTase) fibrillarin utilizes small nucleolar RNAs (snoRNAs), so-called Box C/D snoRNAs that guide the enzyme complex to complementary regions of the rRNA for methylation [2]. Base methylation of rRNA and tRNA as well as 2′-O-ribose methylation of tRNA are carried out by position/sequence-specific methyltransferases independent of snoRNAs. For example, in *E. coli* uracil-5-methyltransferase (trmA) and guanine-7-methyltransferase (yggH/trmB) methylate specific bases in tRNA such as uridine 54 (m5U54) or guanosine 46 (m7G46), respectively [3–5]. In

contrast, the Gm18-2′-O-methyltransferase (spoU/trmH) methylates the 2′-O-position of a conserved guanosine at position 18 of tRNA (Gm18) [6].

Interestingly, also messenger RNA (mRNA) is internally modified and carries N6-methyladenosine (m⁶A) [7]. m⁶A is present in mRNA of all higher eukaryotes tested, including mammals, plants, and insects. This modification occurs on average at 1–3 residues within a defined sequence context (e.g., GGACU) per typical mammalian mRNA molecule [7]. Recently it has been reported that m⁶A sites are enriched near stop codons and in 3′ UTRs suggesting an important role in regulation of gene expression [8, 9].

Random RNA modifications may also occur by oxidation through reactive oxygen species (ROS) which are involved in killing of bacteria and cell signaling pathways [10–12]. Over 20 different purine and pyrimidine modifications formed by reactive oxygen species are known; however, 8-hydroxyguanosine (8-OHG) is the most prominent modification [13]. Of note, 8-hydroxyguanosine modification in mRNA leads to reduced protein levels and altered protein function due to ribosome stalling [14]. Interestingly, age-associated oxidative damage to RNA has been demonstrated in neurons and may play a role in neurodegeneration and other diseases [15].

Bacterial and viral RNA are potent stimulators of the innate immune system leading to immune activation [16]. RNA is recognized in the endosome by Toll-like receptors (TLR). TLR3 recognizes double-stranded viral RNA and mRNA, whereas TLR7 and TLR8 sense single-stranded RNA [17]. In contrast, cytoplasmic detection of viral RNA is mediated by the RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) [18]. RIG-I recognizes 5′ triphosphate RNA [19, 20], whereas MDA5 is activated by higher-order RNA structures generated during viral infection [21].

The effect of ribonucleoside modifications on immunostimulation has been investigated only recently. RNA modifications such as 2′-O- or base methylation and the occurrence of pseudouridine negatively modify the immunostimulatory potential of in vitro transcribed RNA with respect to TLR3, TLR7, and TLR8 [22]. Also pseudouridine-containing or 2′-O-methylated synthetic RNA loses its immunostimulatory capacity via RIG-I [19]. We and others have further demonstrated that 2′-O-methylation of synthetic RNA and tRNA not only renders TLR7 ligands non-immunostimulatory but also converts modified RNA into a TLR7 antagonist [23–25].

Eukaryotic mRNA is not recognized by RIG-I or MDA5 due to a 5′ cap structure methylation at the N7 position of the capping guanosine residue (cap 0), and additional 2′-O-methylation(s) at the 5′-penultimate residue (cap 1) and sometimes also at adjoining

residue (cap 2). Interestingly, some viruses that replicate in the cytoplasm (e.g., picornaviruses and coronaviruses) encode functions associated with the formation of a 5′ cap, which are homologous to those found in eukaryotic cells and also support immune evasion. Accordingly, 2′-O-methylation of viral mRNA cap structures by virus-encoded methyltransferases prevents recognition by MDA-5 or host restriction by interferon-induced proteins with tetratricopeptide repeats (IFIT) family members [26, 27].

In summary, the detection and characterization of modified ribonucleosides are important for understanding mechanisms of RNA-induced immune activation or immune evasion.

2 Materials

2.1 HPLC

1. HPLC system with a column heater and UV monitor. We use a Dionex UltiMate 3000 HPLC with autosampler (WPS-300SL), UV-Detector (VWD-3400RS), and column heater (TCC-300SD). Analysis is performed by the Chromeleon 6.80 SR10 Build 2818 software.

2. HPLC column used for separation is an analytical silica based octadecyl end-capped 25 cm × 4.6 mm, 5 μm HPLC column. A silica based 2 cm octadecyl end-capped column served as a guard column.

3. Buffer A: 5 mM ammonium acetate, pH 6.0 (*see* **Note 1**).

4. Buffer B: 40 % (v/v) acetonitrile.

5. Buffer C: 66 % (v/v) methanol.

6. Buffer D: 40 % (v/v) methanol (*see* **Note 2**).

2.2 Standard Nucleosides

	Stock solution (mM)	Working solution (μM)
Cytidine	10	30
Uridine	10	30
2′-Deoxycytidine	10	70
Xanthosine	1	30
2′-O-Methylcytidine	10	30
Inosine	10	30
Guanosine	1	20
7-Methylguanosine	0.5	20

(continued)

	Stock solution (mM)	Working solution (μM)
8-Hydroxyguanosine	3	30
2′-O-Methyluridine	10	30
2′-Deoxyguanosine	1	15
Thymidine	10	200
2′-O-Methylguanosine	1	20
N_1-Methylguanosine	1	20
N_2-Methylguanosine	1	50
Adenosine	10	30
2′-Deoxyadenosine	10	80
2′-O-Methyladenosine	10	50
5′-Methyldeoxycytidine	5	50
5′-Methyluridine	10	50
N_6-Methyladenosine	1	10
N_6N_6-Dimethyladenosine	10	80

For solubilization *see* **Note 3**.

2.3 RNA Digestion

1. 10 mM Zinc chloride.
2. 300 mM Ammonium acetate, pH 5.3.
3. Tris Base: 100 mM, pH 8.3.
4. Magnesium acetate: 10 mM (*see* **Note 4**).
5. P1 endonuclease from *Penicillium citrinum*, SvP (Snake venom phosphodiesterase from *Crotalus adamanteus*, Sigma, cat. number P3134, 0.5 U/ml), and alkaline phosphatase (AP).
6. 50 µg RNA of interest (*see* **Note 5**).

2.4 Competitive ELISA for 8-Hydroxyguanosine (8-OHG)

1. Phosphate buffered saline (PBS).
2. Washing buffer: PBS + 0.05 % Tween20, store at RT.
3. Blocking buffer 1: washing buffer + 1 % bovine serum albumin fraction V (BSA), store at 4 °C.
4. Blocking buffer 2: blocking buffer 1 with addition of 2 % Sucrose and 0.05 % Casein hydrolysate, store at 4 °C.
5. Substrate buffer: 65 mM disodium hydrogen phosphate, 35 mM citric acid, pH 5.0, store at 4 °C.
6. Substrate: 20 ml substrate buffer + 20 mg O-Phenylenediamine dihydrochloride (OPD) + 20 µl 30 % H_2O_2. Store at 4 °C. Light sensitive, stable for 2 h.

7. Antibodies: Mouse Anti-8-OHG monoclonal antibody, Clone 15A3 (Cayman, cat. number 10011446, 0.65 mg/ml), store at –20 °C. Goat anti-mouse IgG, peroxidase-conjugated, (Jackson cat. number 115-035-062, 0.8 mg/ml), store at 4 °C.

8. Standard: 8-OHG (Cayman, cat. number 89300, 10 mg/ml, nucleoside concentration); Store at –20 °C.

9. BSA conjugated 8-hydrogyguanosine (OHG) (8.45 mg/ml, protein concentration) (*see* **Note 6**).

10. Photometer: Emax (Molecular Device).

11. Microtiter plates: MaxiSorp 96 well (Nunc).

3   Methods

3.1   Analytical HPLC   For detection of modified nucleotides in a given RNA sequence HPLC analysis of digested RNA is a suitable method with high sensitivity and specificity. Before starting the analysis, standard mixtures of nucleosides should be analyzed with the standard protocol to yield high resolution and reproducibility (*see* **Note 7**) (Fig. 1). The occurrence of RNA modifications within a RNA sample of interest (e.g., total RNA from a eukaryotic cell line) can be judged by comparison and overlay of the individual chromatograms (Fig. 1).
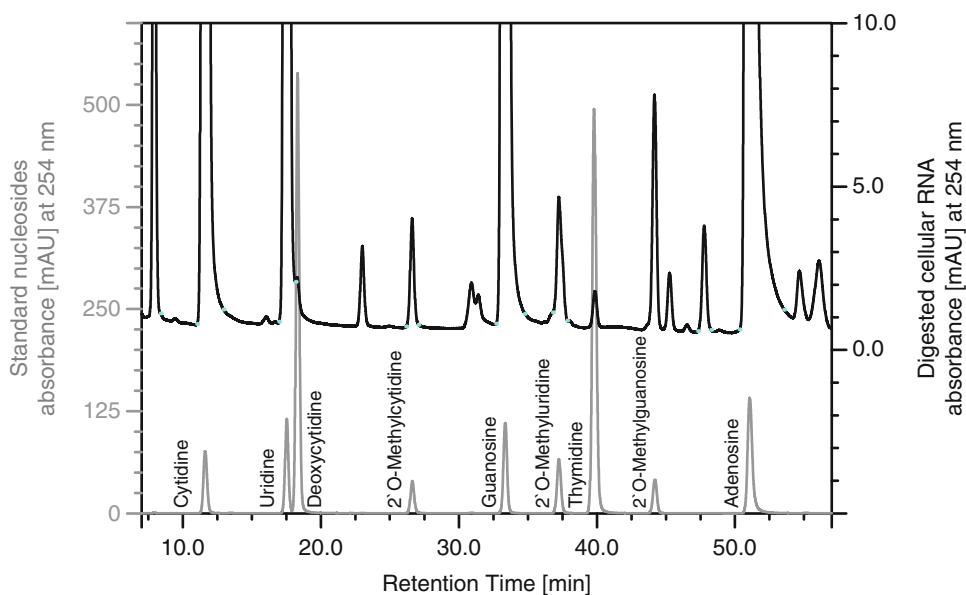

Fig. 1 Chromatogram overlay of standard nucleosides cytidine, uridine, 2′-deoxycytidine, 2′-O-methylcytidine, guanosine, 2′-O-methyluridine, thymidine, 2′-O-methylguanosine, and adenosine (*grey*) and nucleosides recovered from a total RNA preparation of the epithelial colorectal adenocarcinoma cell line Caco-2 (*black*). Separation was performed at a linear gradient of 0–20 % buffer B over 50 min with a flow rate of 0.85 ml/min. Column temperature was set to 21 °C

3.1.1 Standard Protocol

1. Set column oven temperature to 21 °C.

2. Set flow rate to 1 ml/min.

3. Equilibrate column with 1 column void volume of buffer A.

4. Decrease flow rate to 0.85 ml/min.

5. Load column with standard nucleosides or digested and dephosphorylated RNA (*see* Subheading 3.2).

6. Run a linear gradient from 0 to 25 % buffer B over 50 min.

7. Run 100 % buffer B for 3 column void volumes and decrease flow rate to 0.5 ml/min within 2 min in parallel.

8. Equilibrate with 3 column void volumes of buffer A and increase flow rate to 1 ml/min within 8 min.

3.1.2 Modified Protocol to Separate Nucleosides with Similar Characteristics

The separation of modified nucleosides can be limited when the nucleoside characteristics are very similar (e.g., 2′-O-methylguanosine and N1-methylguanosine). Therefore, the HPLC run conditions have to be optimized. By varying temperature and gradient slope it is possible to efficiently separate 2′-O-methylguanosine and N1-methylguanosine which is not achieved by the standard protocol (Fig. 2) (*see* **Note 8**).

1. Set column oven to 8 °C.
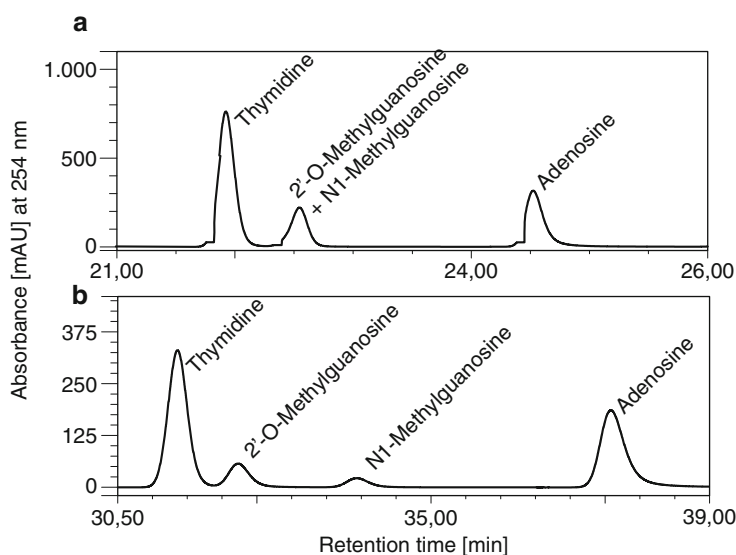
2. Set flow rate to 0.85 ml/min.

Fig. 2 (**a**) Chromatogram with the standard nucleosides thymidine, 2′-O-methylguanosine, N1-methylguanosine, and adenosine. The gradient was set from 0 to 60 % buffer B over 66.5 min. Column temperature was set to 37 °C. (**b**) Chromatogram with the standard nucleosides from (**a**) with a buffer B gradient from 7 to 17.4 % over 35 min and a flow rate of 0.85 ml/min. Column temperature was set to 8 °C

3. Equilibrate column with 1 column void volume of buffer A.

4. Load column with selected standard nucleosides or digested and dephosphorylated RNA.

5. Run a linear gradient from 7 to 17.4 % buffer B over 35 min.

6. Decrease flow rate to 0.5 ml/min.

7. Run 100 % buffer B for 3 column void volumes.

8. Equilibrate with 3 column void volumes of buffer A.

3.1.3 UV Spectra for Discrimination of Nucleosides

The nucleoside specific UV spectra are an additional characteristic that can be used to discriminate nucleosides. For example, adenosine, guanosine, and 8-hydroxyguanosine differ in the wavelength of maximum absorbance and number of peaks (Fig. 3a).
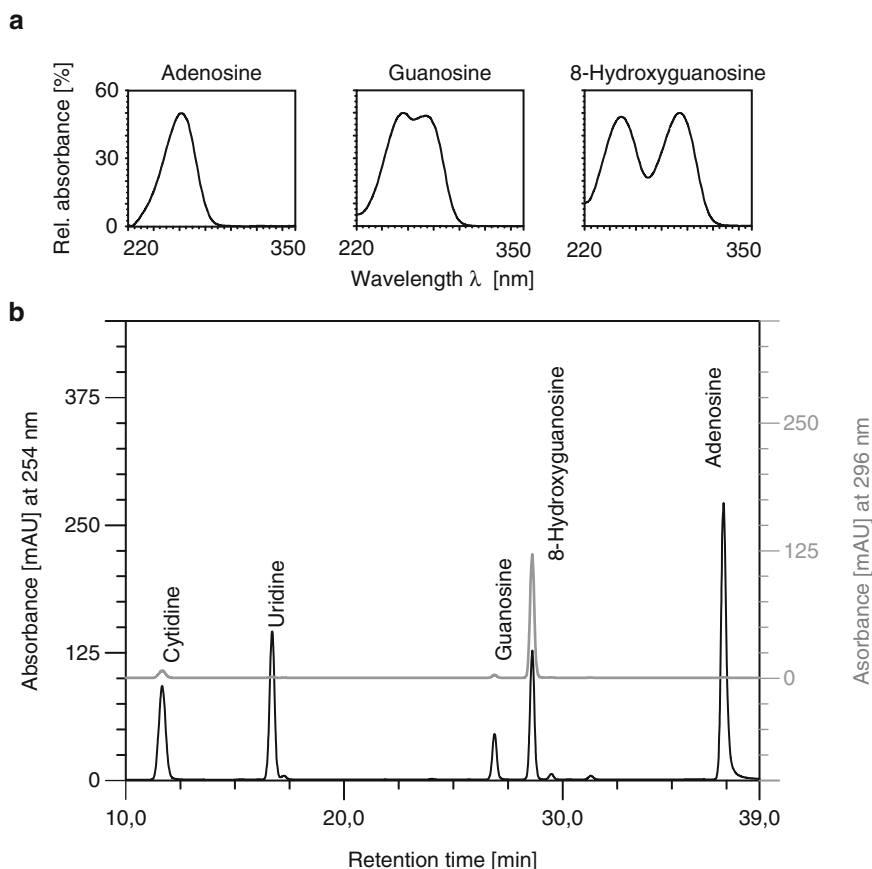

Fig. 3 (**a**) UV-spectrum of adenosine with one distinct absorption maximum at 261.0 nm, of guanosine with two nearby relative maxima at 255.9 and 274.0 nm and of 8-hydroxyguanosine with maxima at 248.2 and 294 nm. (**b**) Chromatogram overlay of nucleosides containing a mixture of guanosine, cytidine, uridine, adenosine, and 8-hydroxyguanosine. Absorbance at 254 nm (*black*) and 296 nm (*grey*) were detected in parallel. The column temperature was set to 21 °C and separation was performed at a linear gradient of 0–25 % buffer B 50 min with a flow rate of 0.85 ml/min

A suitable UV detector connected to the HPLC system allows to record UV spectra and retention time simultaneously. Using the following protocol 8-hydroxyguanosine, which has two absorbance maxima at 248 and 294 nm, can be distinguished from guanosine by retention time and UV-absorbance (Fig. 3b).

1. Set column oven to 21 °C.
2. Set flow rate to 1 ml/min.
3. Equilibrate column with 1 column void volume of buffer A.
4. Set flow rate to 0.85 ml/min.
5. Load column with selected standard nucleosides or digested and dephosphorylated RNA.
6. Run a linear gradient from 0 to 20 % buffer B over 50 min.
7. Decrease flow rate to 0.5 ml/min.
8. Run 100 % buffer B for 3 column void volumes.
9. Increase flow rate to 1 ml/min.
10. Equilibrate with 3 column void volumes of buffer A.

3.1.4 Quantification of Nucleosides

Quantification can be achieved using the linear standard plot method [28].

1. Dilute 500 μM of the relevant nucleoside 1:1 for ten times. Subject these standards to HPLC analysis and plot the values of the peak areas against the concentration to create a linear standard curve.
2. The software Chromeleon 6.80 SR10 Build 2818 can determine the peak area of the relevant nucleoside in the sample of interest and calculate the absolute amount using the standard curve.

3.2   RNA Digestion

For HPLC analysis the RNA of interest has to be cleaved into nucleotides by nuclease P1 and snake venom phosphodiesterase (SvP) with subsequent dephosphorylation by alkaline phosphatase (AP) to obtain nucleosides [29, 30].

1. Dissolve 2–100 μg RNA in 60 μl H_2O.
2. Add 10 μl ammonium acetate, 20 μl zinc chloride, and 5 μl of P1 endonuclease and incubate at 37 °C overnight or 42 °C for 2 h.
3. Add 15 μl of Tris buffer and 15 μl magnesium acetate.
4. Use 2.5 μl SvP and 0.25 μl AP and incubate at 37 °C for 2 h. After dephosphorylation, centrifuge at $30,000 \times g$ for 10 min and harvest the supernatant carefully. Adjust the volume to 130 μl with buffer A and use for HPLC injection.

3.3 Competitive 8-Hydroxyguanosine ELISA

The unknown 8-OHG samples or 8-OHG standards are added to an 8-OHG/BSA conjugate preabsorbed microtiter plate. Then an anti-8-OHG monoclonal antibody is added with subsequent detection by a secondary peroxidase-labeled antibody. The 8-OHG content in unknown samples is determined by comparison with the 8-OHG standard curve [31].

1. Dilute 8-OHG-BSA conjugate 1:200,000 in PBS. Coat each well of the microtiter plate with 50 μl. Store at 4 °C overnight.

2. Wash each well six times with 250 μl washing buffer.

3. Block plates with 250 μl/well blocking buffer 2. Incubate for 2 h at RT and wash six times.

4. Prepare the RNA in a twofold dilution series six times starting with 20 μg/ml in PBS. Dilute the 8-OHG standard 1:1 in PBS for 12 times starting at 2 μg/ml. Add 50 μl of standards and samples to the wells. Use three replicates for each sample and duplicates for standard (*see* **Note 9**).

5. Immediately add 50 μl of 8-OHG antibody diluted 1:10,000 in blocking buffer 1 to each well. Incubate for 1 h at RT.

6. Wash the plate six times and add 50 μl of peroxidase-conjugated goat anti-mouse IgG diluted 1:20,000 in blocking buffer 1. Incubate for 1 h at RT.

7. Wash the plate six times and add 50 μl substrate solution per well. Incubate for approximately 30 min in the dark and terminate the reaction by adding 25 μl 2 M H_2SO_4 to each well.

8. The absorbance is measured at 490 nm against 650 nm used as reference wavelength.

4 Notes

1. For preparing buffer A dissolve 0.385 g of ammonium acetate in 50 ml H_2O and adjust pH value with 10 % glacial acetic acid to pH 6.0. To sterilize solution use a 0.2 μm disposable filter (Kobe, Germany) and filtrate into 950 ml HPLC grade H_2O.

2. For all buffers HPLC gradient grade solution should be used. Adhere to professional storage, handling, and disposal of buffers. Buffers can be stored at room temperature. All chemicals used for buffers were supplied from Carl Roth, Germany.

3. Guanosine and methylated derivatives should be dissolved in buffer A whereas adenosine and methylated derivatives are more soluble in H_2O. Solubility is enhanced by increasing temperature up to 50 °C and ultrasonic treatment. Other nucleosides can be dissolved in H_2O or buffer A. For long-term storage, aliquot nucleosides and freeze at –20 °C. To set up working

solutions, thaw aliquots and mix nucleosides in buffer A to yield working concentrations listed in Subheading 2.2.

4. All buffers for RNA digestion should be sterilized by filtration (0.2 μm filter) and stored at 4 °C. Chemicals were supplied from Carl Roth, Germany.

5. Total cellular or in vitro transcribed RNA can be used, but should be phenol–chloroform-purified before digestion and HPLC analysis. RNA should be stored at –80 °C.

6. All chemicals were supplied by Carl Roth, Germany or as indicated. BSA conjugated 8-OHG was generated as described by Senapathy et al. [32]. Store conjugated protein at –20 °C.

7. HPLC equilibration, separation, and cleaning procedure:

    (a) Set up equilibration: Rinse the column which is stored in buffer C with at least 5 void volumes of buffer C, buffer B and buffer A to equilibrate the column. Take care to remove air bubbles thoroughly before connecting the column.

    (b) Separation: Set column oven temperature and run parameters according to the protocols in Subheading 3.

    (c) Cleaning procedure: Wash column with least 5 void volumes of buffer B followed by the equal amount of buffer C. If the column is not used for more than 1 week, remove and seal it.

8. Change parameters of the standard protocol in the order given to optimize HPLC run such as gradient slope, flow rate, column temperature, eluotropic strength of elution buffer, ion strength of buffer A, pH value of buffer A, use of multidimensional buffer systems [33].

9. For some RNA samples digestion and dephosphorylation before 8-OHG detection may increase sensitivity.

Acknowledgments

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